Vol. III

THE JOURNAL OF NUTRITION

No. 4

JANUARY, 1931

THE EFFECT OF THE USE OF MINERAL OIL UPON THE ABSORPTION OF VITAMIN A*

By

JENNIE I. ROWNTREE

(From the Laboratory of the Department of Pediatrics, State University of Iowa, Iowa City.)

Received for Publication—April 7, 1930

THE use of mineral oil as a laxative has been reported to interfere with the absorption of vitamin A. If this is true, its use should be restricted. Since it is one of the most commonly used laxatives, its effect deserves further study in order to determine whether it is a valuable or pernicious substance.

Dutcher, Ely and Honeywell (1927) fed rats limited amounts of vitamin A and varying amounts of mineral oil and found that growth of rats was prevented when the oil was added to the diet. They are of the opinion, therefore, that mineral oil acts as a solvent for vitamin A, carrying it out of the system. Burrows and Farr (1927) also have reported losses of vitamin A when mineral oil was added to an adequate diet and believe that mineral oil depletes the system of its store of vitamin A. Mellanby (1928) on the other hand believes the effect of mineral oil to be due to interference with the absorption of bile salts. He has shown that bile salts form a stable emulsion with the mineral oil and are, therefore, lost to the system and digestion is impaired when bile salts are not absorbed. He believes that the bile salts act as a hormone for pancreatic secretion or are the vehicles by which secretin is carried through the intestinal wall. In either case there would be little fat digestion or absorption. This is an interesting hypothesis, but Gage and Fish (1924) found in their studies of blood fat that, while there was no evidence of mineral oil absorption from the intestine, butter taken with the mineral oil increased the fat

* The material in this paper is taken in part from a dissertation submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy in Nutrition, Department of Child Welfare, State University of Iowa, 1929.

content of the blood. This would not be in accord with Mellanby's hypothesis. While it is not a foregone conclusion that impaired fat absorption would be accompanied by impaired vitamin A absorption, the work of Bloch (1924) would tend to show that this is true.

The effect of mineral oil may be attributed to other causes than avitaminosis. Sano (1927) studied the effect of diarrhea on the length of time required to deplete the rat of its store of vitamin A and found that prostration was often marked and death common before there were any indications of ophthalmia. Large doses of mineral oil may exert a depressing effect on the secretion of the digestive juices or prevent the enzymes from attacking the food without having any specific effect upon the digestion and absorption of vitamin A.

In the work cited above, the animals were fed large amounts of mineral oil and very small amounts of vitamin A. Therefore it seemed of interest to feed amounts of mineral oil which would be comparable to the therapeutic dose for the human being along with a more generous allowance of vitamin A.

Procedure

A series of experiments was carried on in which rats of different ages and in different nutritive conditions were fed diets high in vitamin A or diets in which the vitamin A was just sufficient for growth. The mineral oil was given in quantities equivalent to one, two and three tablespoons for the human being. It was estimated that five per cent of the dried human diet would be equivalent to two tablespoons of mineral oil. Five per cent of the diets of these rats was approximately half a cubic centimeter daily, and this amount, therefore, approximates two tablespoons for the human being.

Experiment I. Effect of Addition of Varying Amounts of Mineral Oil and Cod Liver Oil to Diets of Xerophthalmic Rats of Different Ages.

In the first part of this experiment a group of rats was used which were late in developing xerophthalmia owing to a change in the stock diet. The average number of days on the vitamin A-free diet before xerophthalmia became evident was forty-six, and the average weight 190 grams. At this time they were given thirty milligrams (one drop) of cod liver oil and 0.25 cc. of mineral oil daily, the latter being approximately two and a half per cent of their diet. Both oils were thoroughly mixed with the basal ration. This amount of cod liver oil contains sufficient vitamin A for growth, and the growth and improvement of the controls is additional evidence.

The results are given in Table I. Seven of the nine rats fed the cod liver and mineral oil immediately resumed their growth though the eyes did not clear in every case. One (No. 134) which was in a deplorable condition before xerophthalmia was evident, showed only a slight improvement in condition and gained only five grams. One (No. 108) lost weight from the time the mineral oil was administered, eliminated a bloody urine, and died in twenty days. One kidney was greatly enlarged and the condition was diagnosed by Dr. Hansmann of the Pathology Department as infected hydronephrosis.

TABLE I

EFFECT OF ADDITION OF MINERAL AND COD LIVER OILS TO DIET OF RATS WITH XEROPHTHALMIA

Variation in diet	Rat	Sex	Days on A-free diet	Weight when depleted	Mineral oil daily	Cod liver oil daily	Days given	Gain or loss
				gms.	cc.	cc.		gms
Both	132W	M	53	200	0.25	0.03	15	44
mineral	122X	M	55	205	0.25	0.03	15	38
and cod	120X	M	55	210	0.25	0.03	15	25
liver oil	134W	M	56	210	0.25	0.03	15	20
	106G	M	54	175	0.25	0.03	15	55
	108G	F	56	178	0.25	0.03	15	−30**
	155Q	F	50	160	0.25	0.03	15	30
	136U	M	52	160	0.25	0.03	15	30
	163P	M	52	180	0.5	0.03	15	42
Cod liver	141R	M	50	200	None	0.03	15	40
oil only	107G	M	56	188	"	0.03	15	37
Mineral	128W	M	44	190	0.25	None	15	−45
oil only	136U	M	42	200	0.25	"	15	−40*

* One drop cod liver oil daily given at this point.

** Died of infected hydronephrosis

It is evident that this amount of mineral oil interfered little with the gain of xerophthalmic rats when a generous amount of cod liver oil was given at the same time. If the one rat with the infected kidney is eliminated, the average weekly gain of those receiving the mineral and cod liver oil was sixteen grams; of those receiving the cod liver oil without the mineral oil, seventeen grams; while those fed the mineral oil without the addition of vitamin A lost nineteen grams weekly. After a loss of

forty grams when mineral oil alone was added, one of the controls (No. 136U) was given a drop of cod liver oil daily, and the same amount of mineral oil as previously. His response was slow, because xerophthalmia had become steadily worse during the time the mineral oil was given, but during the second week the cod liver oil produced a gain of thirty grams. As mentioned above, the rats used in this experiment were late developing xerophthalmia owing to a change in the stock diet on which they were reared. Since Sherman (1926) has shown that rats which are large before they develop xerophthalmia require more vitamin A than smaller animals, it does not appear that the mineral oil dissolved, absorbed, or carried off any of the vitamin A from the one drop of cod liver oil fed these rats.

TABLE II

EFFECT OF ADDITION OF VARYING AMOUNTS OF MINERAL OIL TO DIET OF RATS ADEQUATE IN VITAMIN A

Rat	Sex	Days on A-free diet	Weight when depleted	Mineral oil daily	Cod liver oil daily	Days given	gain	Av. fecal wt. daily
			gms.	cc.	cc.		gms.	gms.
268K	F	40	90	0.25	0.03	25	56	0.70
259J	F	41	110	0.25	0.03	25	64	0.66
240G	M	47	120	0.50	0.03	25	66	0.84
234G	M	48	122	0.75	0.03	25	84	1.5
262J	M	45	95	0.75	0.06	25	70	1.12
274K	F	43	112	0.75	0.06	25	65	1.00
225E	F	45	104	0.75	0.06	25	58	0.9
228E	M	43	136	0.75	0.06	25	54	1.0
253I	M	47	106	None	0.03	25	65	0.55
286N	M	30	82	"	0.03	25	68	0.5
229E	F	45	96	"	0.03	25	55	0.5

Since these rats were so nearly grown before xerophthalmia appeared, it seemed wise to repeat the test using more nearly standard rats—those depleted in five to six weeks and weighing 110 grams at the time. The amount of mineral oil given varied from two and one-half to seven and a half per cent of the ration of the rats (0.25 cc. to 0.75 cc.). These amounts would correspond to therapeutic doses of one and three tablespoons. The results are given in Table II.

These results show that when there is an adequate amount of vitamin A in the diet, mineral oil in doses comparable to the therapeutic have no effect on the absorption of vitamin A. The average gain of the five rats fed 0.75 cc. of mineral oil daily was 2.6 grams. This amount of oil would compare with three tablespoons for the human being. The controls receiving no mineral oil but the same amount of cod liver oil made an average gain of 2.5 grams daily. The food intake of the rats fed the mineral oil was very nearly the same as the controls, nine to ten grams daily, but the feces were very different both in amount and nature. The average daily weight of the feces of the five fed 0.75 cc. of mineral oil was 1.1 grams; of those fed 0.25 cc. of oil, 0.68 grams; and of those fed the cod liver oil only, 0.52 grams. The feces of those fed the mineral oil were large, light colored, well formed, and had an oily appearance. Since the increase in fecal weight was no greater than the mineral oil ingested, it does not appear that there was undue loss of food through the feces. It seemed possible that the light color of the feces might be due to undigested starch, but no color was produced when tested with iodine.

Experiment II. It seemed of interest to determine the effect of mineral oil on the growth of rats that had been receiving only enough vitamin A to make moderate gains. A group of eight rats was used, therefore, which had been previously depleted of vitamin A for another experiment and which was receiving at the time only enough vitamin A to make gains of two to three grams daily. The source of vitamin A was not identical but in every case litter mates receiving the same extract and making similar previous gains were used as controls. The experimental rats were given five per cent of mineral oil and the controls the same diet without the mineral oil.

Results. Table III shows that of the five rats given the mineral oil, four died within a month while the fifth only maintained its weight. The latter probably had previously stored more vitamin A in its tissues. The three litter mate controls continued to gain very slowly. This premature flattening of the growth curves of the controls indicated that an insufficient amount of vitamin A was fed. From this it appears that rats receiving just enough vitamin A for moderate growth are unable to survive when comparatively large amounts of mineral oil are added to the ration. Whether their death was due to vitamin A deficiency or poor absorption is not certain. There was loss of appetite, and on autopsy the alimentary canal was empty. Xerophthalmia in these rats was not as pronounced as the respiratory difficulties.

TABLE III

EFFECT OF ADDITION OF FIVE PER CENT OF MINERAL OIL TO DIET OF RATS RECEIVING SUFFICIENT VITAMIN A IN FOOD EXTRACTS TO PRODUCE MODERATE GAINS

Rat	Sex	Initial weight	Previous weekly gain	Mineral Oil		Gain or loss	Remarks
				cc. daily	Days given		
			gms.			gms.	
60F	F	170	10	0.5	17	−48	Died
63F	M	140	10	0.5	31	−32	"
50H	M	206	15	0.5	16	−56	"
52H	M	200	14	0.5	27	−66	"
98M	F	140	25	0.5	33	0	"
69G	M	228	24	0.5	35	0	"
Controls							
64F	F	172	11	0	31	6	
51H	F	186	16	0	16	12	
68G	F	165	12	0	35	15	

DISCUSSION

These results are surprising when the findings of other workers are considered. Dutcher, Ely, and Honeywell (1927) found that two and a half parts of mineral oil to one of butter killed their animals. Yet in the experiment reported above, as much as twenty-five parts of mineral oil to one part of cod liver oil were given and the rats grew at a normal rate. Moreover, the tests were made on rats depleted of their vitamin A store with xerophthalmia fully established. Since Dutcher and his associates fed but 0.0666 and 0.1133 grams of butter daily, it would appear that the vitamin A allowed was minimal. Nelson and Jones (1928) have reported that butter is about one-fifteenth as potent a source of vitamin A as cod liver oil, and this would mean that Dutcher was feeding the equivalent of but four and eight milligrams of cod liver oil daily. It is generally conceded that ten milligrams is the minimum amount to provide the necessary vitamin A for the rat when the best cod liver oils are used. It would seem, therefore, that their results are not unlike those in the last test reported here in which the animals had been fed just enough vitamin A to make moderate gains, but were unable to maintain growth when mineral oil was added to their rations.

Burrows and Farr (1927) fed mineral oil equal to fourteen and seven per cent of the diet of the rats, and found that young rats were unable to withstand the mineral oil but the older ones were not affected. All the animals used in the above experiments were from two to three months of age, except the lactating mothers in the last experiment.

In all except the first test it should be noted that the vitamin A was probably considerably more than the minimum for normal growth; but thirty milligrams, one drop daily, is not an excessive amount for an animal previously depleted of vitamin A. It seems, therefore, that the effect of mineral oil is dependent on the quantity of vitamin A in the diet. If sufficient vitamin A is provided, no harmful effects can be noted. If a minimum only is given, the rats succumb.

Conclusions

The effect of mineral oil on the absorption of vitamin A appears to be dependent on the amount of vitamin A in the diet. When the quantity of mineral oil given to rats is comparable to the human therapeutic dose and the vitamin A only enough to produce subnormal growth, the rats are unable to withstand the effects of the mineral oil. However, if the amount of vitamin A is adequate, mineral oil can be given without noticeable effects. If sufficient vitamin is given, the amount of mineral oil could be increased fifty per cent without producing a slackening of growth or preventing the cure of severe ophthalmia. It is suggested that if this laxative is employed, it should be accompanied by a generous amount of fat-soluble vitamins.

BIBLIOGRAPHY

1. Bloch, C. E., 1924, *Amer. Jour. Dis. Children*, XXVIII, 659–667.
2. Burrows, M. T., and Farr, W. K., 1927, *Proc. Soc. Exp. Biol. and Med.*, XXIV, 719–723.
3. Dutcher, R. A., Ely, J. O., and Honeywell, H. E., 1927, *Proc. Soc. Exp. Biol. Med.*, XXIV, 953–955.
4. Gage, S. H., and Fish, P. A., 1924, *Amer. Jour. Anat.*, XXXIV, 1.
5. Mellanby, M., 1928, *Jour. Physiol.*, LXIV, 331–340.
6. Nelson, E. M., and Jones, D. B., 1928, *Jour. Biol. Chem.*, LXXX, 215–226.
7. Sano, T., 1927, *Jour. Pediat.* Tokyo, June 1927, 800. Abst. by Kato in *Amer. Jour. Dis. Children*, XXXIV, 1063.
8. Sherman, H. C., and Cammack, M. L., 1926, *Jour. Biol. Chem.*, LXVIII, 69–74.

ON THE PREPARATION OF A CONCENTRATED SOURCE OF THE HEAT-LABILE VITAMIN B, FREE FROM CONTAMINATION WITH THE HEAT-STABLE FACTOR G*

By

HERBERT M. EVANS AND SAMUEL LEPKOVSKY,

(From the Department of Anatomy, University of California, Berkeley.)

Received for Publication—April 24, 1930

INTRODUCTION

SINCE the establishment of the existence of two water-soluble vitamins, one heat-labile and one heat-stable (1), it becomes necessary, when only one variable is desired, to feed these vitamins separately. English workers (2, 3, 4) have prepared vitamin B free from the heat-stable factor in yeast by using various precipitants (lead acetate, barium hydroxide, acid mercuric sulfate) and final adsorption on charcoal. We desired to secure such a preparation with the elimination, in so far as was possible, of procedures involving bulky precipitates, particularly metallic sulfides, because of the loss of vitamin B in such precipitates. It was our hope that starting with a material rich in vitamin B but poor in the heat-stable factor, we could attain the same end with fewer manipulations. We found the outer layers of the rice kernel particularly suitable for our purposes, since they are very rich in the heat-labile and relatively poor in the heat-stable factor. Starting with this material, attempts were made to obtain vitamin B free from the heat-stable factor.

EVALUATION OF THE VARIOUS OUTER LAYERS OF THE RICE KERNEL FOR THEIR CONTENT OF VITAMIN B AND THE HEAT-STABLE, WATER-SOLUBLE VITAMIN

The outer layers of the rice kernel are milled off in three stages. The outer layer is known to the trade as hullers first-break bran; then comes an inner layer known as hullers second-break bran; and finally the layer known as polish (5).** For convenience we shall refer to these layers as rice bran No. 1, for the outermost layer, rice bran No. 2, for the next layer,

* Aided by grants from the Committee for Research in Problems of Sex of the National Research Council and from the Rockefeller Foundation, and from the Bureau of Dairying of the United States Department of Agriculture. These funds have been generously augmented by the Board of Research and by the College of Agriculture of this institution.

** We are greatly indebted to Rosenberg Bros., San Francisco, for the rice polish and rice bran used in this investigation.

and we shall retain for the innermost layer the trade name "rice polish." Much of our work has been done with the combination of rice bran No. 1 and rice bran No. 2. This combination we shall refer to simply as "rice bran."

The vitamin content of each layer of the rice kernel was determined by feeding an extract prepared with 25 per cent alcohol as follows: 1 kg. of each layer of the rice kernel was shaken several times daily with 4 liters of 25 per cent ethyl alcohol for 1 to 2 days. The alcohol was filtered off and the extraction was repeated twice with 3 liters of fresh 25 per cent alcohol. The separate extracts were combined and concentrated in vacuo to 250 cc. Thus 1 cc. of extract was equivalent to 4 gm. of the original material.

The procedure used to evaluate vitamin B has been previously described (6). Diet 540 was used when materials were tested for their content of the heat-stable factor and Diet 542 when tests were made for the heat-labile factor.

Diet 540		*Diet 542**	
Casein (L III)	25.0	Casein (L III)	20.0
Sucrose	75.0	Sucrose	70.0
Salts No. 185	4.0	Autoclaved yeast	10.0
		Salts No. 185	4.0

The diets were supplemented with 2 drops of cod liver oil daily.

Female rats were placed on one or the other of these diets when 21 days old and the various fractions were usually added 10 days later, although in some cases it was several days longer, as is indicated in the legends to the charts. The growth curves presented are for individual animals with the first point representing the 25th day of life. The circles interrupting the curve represent the occurrence of oestrus.

The amount of vitamin B in the 25 per cent alcoholic extracts is shown by the growth curves presented in Chart 1. When 0.1 cc. of extract was fed, the growth was slightly better on rice bran No. 1 than on either rice bran No 2 or rice polish. No differences were detected in the extracts of the various layers when 0.5 cc. of extract was fed, indicating that the vitamin B requirements had been satisfied in all the extracts at this level.

If these measurements are to be significant, we must be certain that most, if not all, of the vitamin has been extracted by the solvent used.

* Casein L III is washed one week with acidulated water and then dehydrated with alcohol and ether. Autoclaved yeast is Fleischmann's yeast autoclaved for 6 hours. We are deeply indebted to the Fleischmann Yeast Company of New York for this excellent source of the heat-stable vitamin.

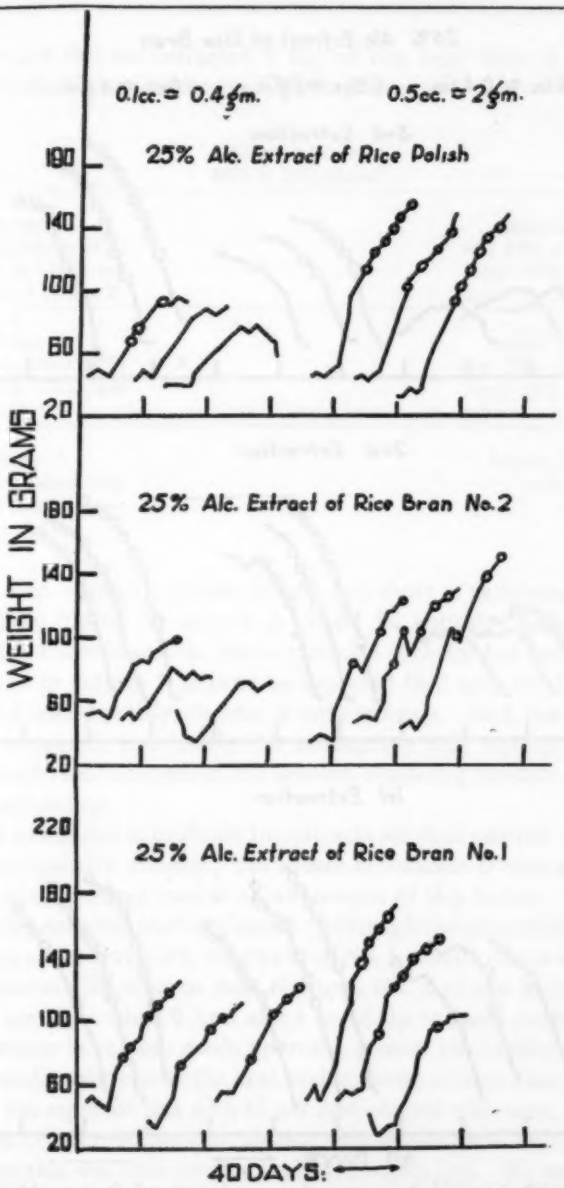

0.1cc. = 0.4 gm.
0.5cc. = 2 gm.
25% Alc. Extract of Rice Polish
25% Alc. Extract of Rice Bran No. 2
25% Alc. Extract of Rice Bran No. 1
WEIGHT IN GRAMS
40 DAYS

CHART 1.—Curves which demonstrate the amount of vitamin B removed by 25% alcohol from the 3 outer layers of the rice kernel. The animals were fed Diet 540 on the 21st day. Diet 542 replaced Diet 540 on the 35th day and the extracts were started on the 40th day.

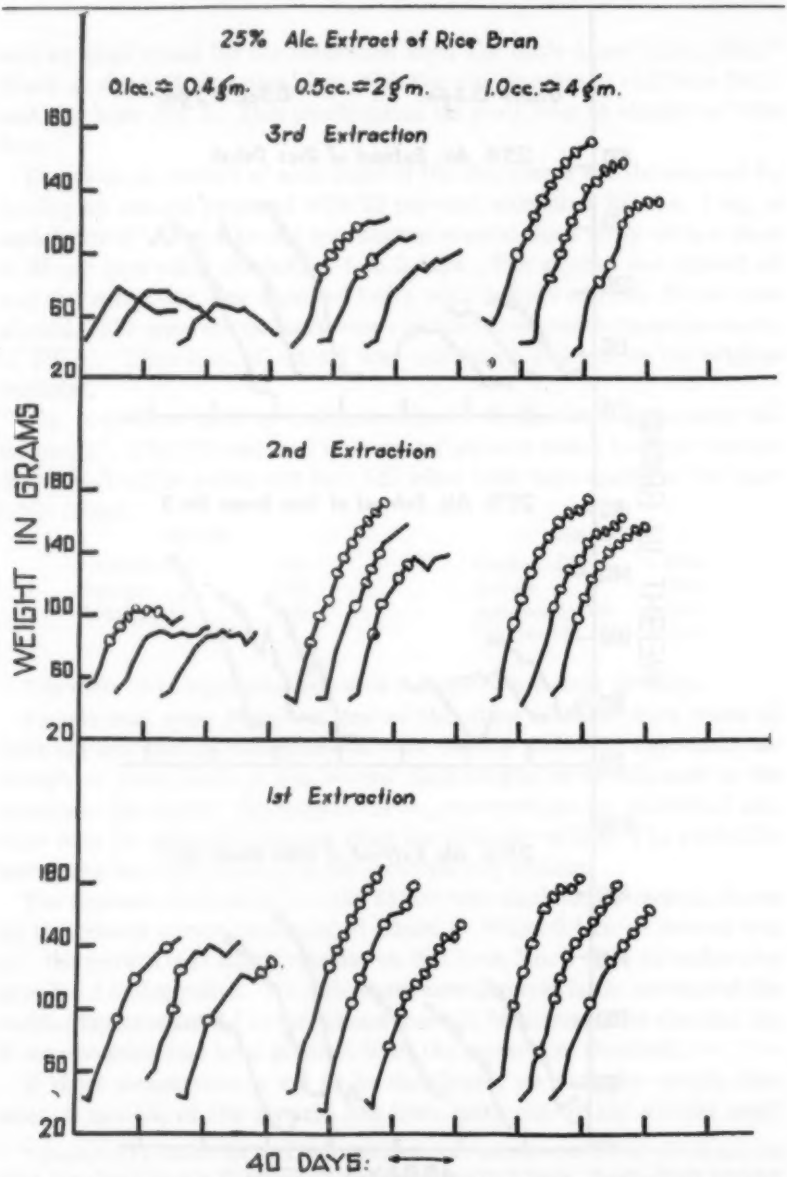
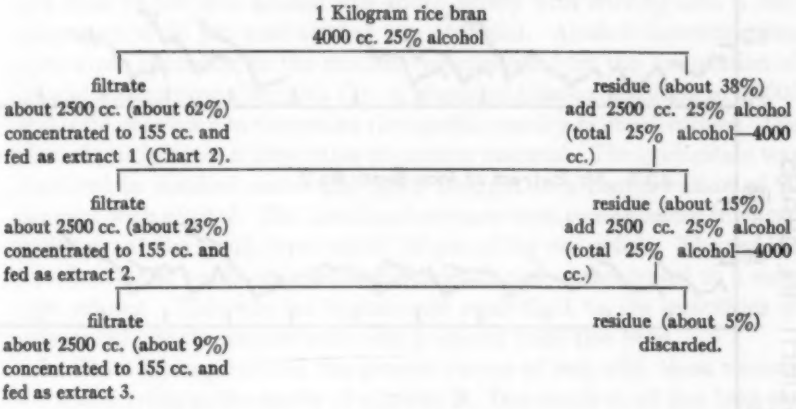


CHART. 2.—Curves which demonstrate the amount of vitamin B removed from rice bran by 3 successive extractions with 25% alcohol. The animals were placed on Diet 540 on the 21st day. On the 30th day Diet 542 replaced Diet 540 and the extracts were fed.

To determine this we extracted 1 kg. of rice bran with 4 liters of 25 per cent alcohol according to the following schema:



Since 0.1 cc. extract 1 (Chart 2) falls just short of satisfying vitamin B requirements, 0.1 cc. of extract 2 should be entirely inadequate, but 0.5 cc. extract should suffice. Since extract 3 contains but one-seventh of the material in extract 1, it is to be expected that only the 1.0 cc. level of extract 3 would satisfy vitamin B requirements. Such results have in fact been obtained, and justify the assumption that vitamin B is thoroughly distributed throughout the solvent, rendering possible practically complete extraction.

We next proceeded to evaluate the extracts for their content of the heat-stable, water-soluble vitamin. No source of vitamin B was added since the extracts themselves carried an abundance of this factor. The results of our studies are presented in Chart 3. Although the growth is poor on all the extracts at the levels fed, the rice bran No. 1 contains more of the heat-stable, water-soluble vitamin than rice bran No. 2 or rice polish. This is especially apparent when 0.5 cc. and 1 cc. of the extracts are fed.

Observations have been made upon the greater insolubility in concentrated alcoholic solutions of the heat-stable, water-soluble vitamin. Goldberger (1) has reported this with 85 per cent alcohol with corn; and Chick and Roscoe (2) with 90 per cent alcohol with wheat germ. We desired to see whether this was true for rice bran and rice polish. We were able to confirm Goldberger's work with 85 per cent alcohol with rice bran and rice polish.

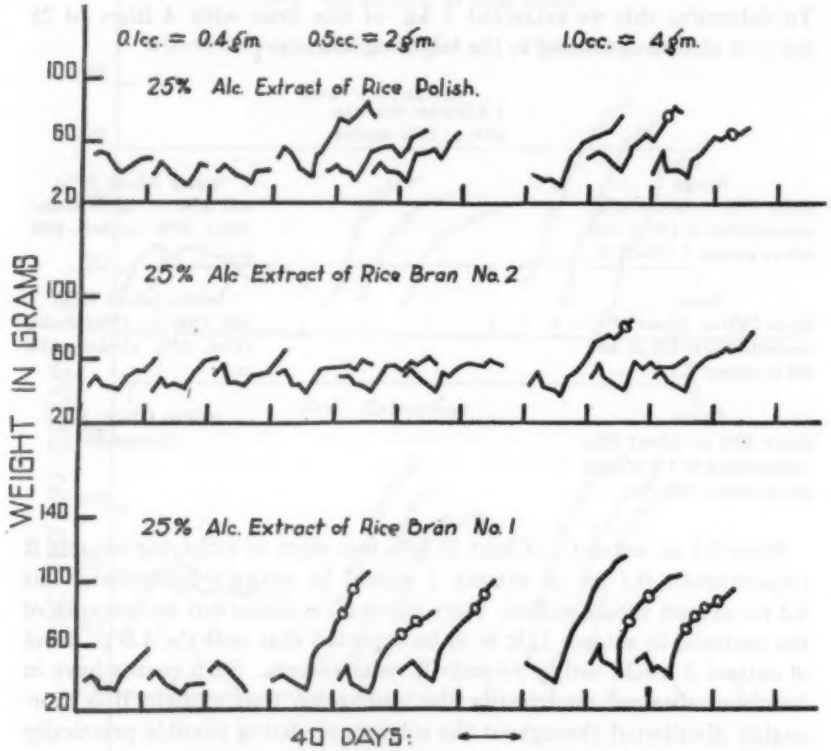


CHART 3.—Curves which demonstrate the amount of the heat-stable, water-soluble vitamin removed by 25% alcohol from the 3 outer layers of the rice kernel. The animals received Diet 540 after the 21st day and the extracts were started on the 45th day.

AN ATTEMPT TO FRACTIONATE VITAMIN B FROM THE HEAT-STABLE, WATER-SOLUBLE VITAMIN

Although excellent results were obtained when rice bran was extracted with 85 per cent alcohol, the results obtained with rice polish were not so encouraging because of losses in vitamin B. Inasmuch as we were particularly interested in the rice polish, because of its initial poverty in the heat-stable, water-soluble vitamin, we decided to combine the thoroughness of extraction of 25 per cent alcohol with the selective action of 90 per cent alcohol. One extraction was made with 25 per cent alcohol in the proportions of 1 kg. of rice polish to 4 liters of 25 per cent alcohol. Since our data tend to confirm our assumption that vitamin B is nearly, if not

entirely, distributed throughout the solvent, we based our calculations upon the rice polish represented in the extract recovered. The 25 per cent alcoholic extract was concentrated in vacuo to a syrup (sp. gr. 1.23–1.27), and then 95 per cent alcohol was added slowly with stirring until a concentration of 90 per cent alcohol was obtained. Alcohol determinations were made according to the method recommended by the Association of Official Agricultural Chemists (7). A Westphal balance, sensitive to 0.0002 or 0.0003, was used to determine the specific gravity of the distillate. The alcohol precipitated a large mass of gummy material. The precipitate was dissolved in distilled water and again brought to a concentration of 90 per cent with alcohol. The combined extracts were concentrated in vacuo until 1 cc. of the syrup represented 10 gm. of the rice polish. The residue was distilled in vacuo to remove the alcohol, and concentrated to a suitable volume. This was fed in amounts equivalent to the quantities of extract fed. Such extracts were also prepared from rice bran.

In Chart 4 are presented the growth curves of rats with these various extracts serving as the source of vitamin B. One-tenth cc. of rice bran extract practically satisfies the rat's requirement for the vitamin B, and although the same equivalent of the residue allows fair growth, it falls considerably short of satisfying this requirement. However, 0.4 cc. of the residue practically satisfies the vitamin B requirement. On this basis, the vitamin B losses in the residue are great, since its vitamin B content is 20 per cent or more of that contained in the extract.

The rice polish extracts behave similarly. The rats do as well on 0.1 cc. as on the higher levels of the extract. Here, too, the losses in the residue are great, but not so great as was the case with rice bran. Very poor growth took place on the residue equivalent to 0.1 cc. of the extract. On 0.65 cc. of the residue, good growth resulted, but not quite so good as on 0.1 cc. extract. Hence, the losses in the rice polish residue were considerable, since its vitamin B content approximated somewhat less than 20 per cent of that contained in the extract.*

At the same time that the vitamin B was being evaluated, simultaneous experiments were carried on to determine the potency of these ex-

* In this connection we wish to point out that rats grow better on the rice bran extracts than on the rice polish extracts. The variable is not vitamin B, for with increasing amounts of rice polish extracts there is no further increase in growth, such as would be the case if vitamin B were the variable factor. It is true that the differences in growth between the rice bran and rice polish extracts are not great, but there is no doubt that a difference does exist. The difference cannot be said to be due to differences in animals, as all the animals came from a large group of young, and were fed at the same time.

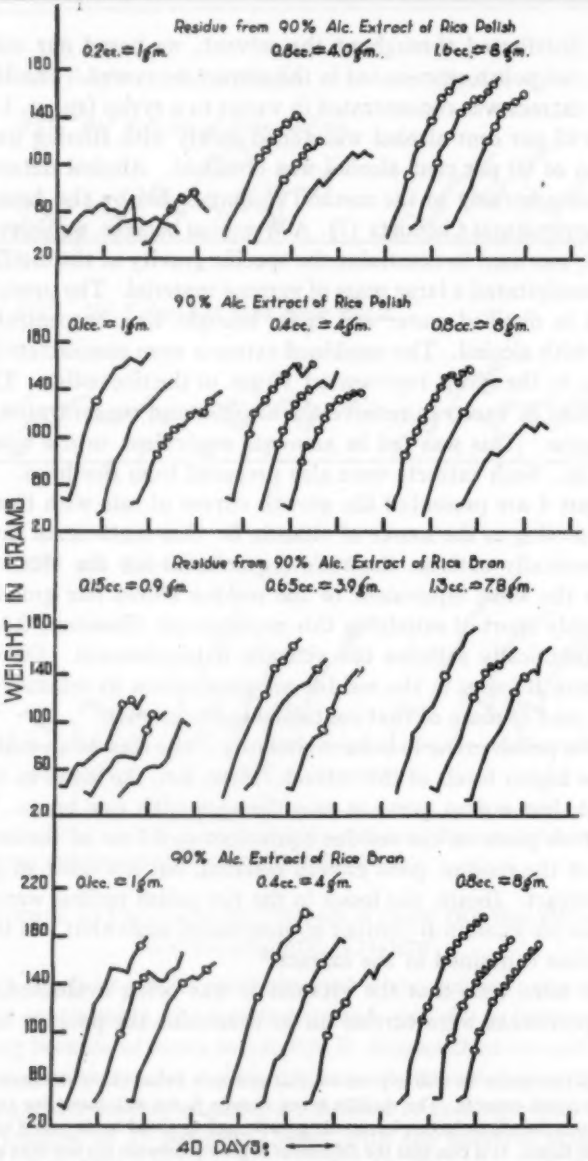


CHART 4.—Curves which demonstrate the amount of vitamin B in a 90% alcoholic extract and residue prepared from a 25% alcoholic extract. Extracts of rice bran and rice polish were used. The animals were maintained on Diet 540 from the 21st to the 33rd day. At this time Diet 542 replaced Diet 540 and the extracts were fed.

tracts for the heat-stable, water-soluble vitamin. The data are presented in Chart 5. Since it is evident that the vitamin B is not a limiting factor (cf. Chart 4) in any of the experiments, it is clear that the heat-stable factor is relatively low in these extracts.

The amount of the heat-stable, water-soluble vitamin in rice bran extract has been greatly reduced by the treatment with 90 per cent alcohol. The animals made poor growth, and showed "pellagric" symptoms at all levels. The fact that the heat-stable, water-soluble vitamin has been precipitated in the residue is shown by the superior growth of rats fed rice bran residue equivalent to 0.4 and 0.8 cc. of extract. Not only is the growth superior, but no "pellagric" symptoms appeared in any of the animals. Hence, 90 per cent alcohol is very efficient in removing the largest part of the heat-stable, water-soluble vitamin.

A similar situation was found with rice polish. The animals grew very poorly on the rice polish extract at all levels, and also showed "pellagric" symptoms. The rice polish residue gave better growth than the extract only at the highest level fed. The growth was somewhat better and no symptoms of pellagra appeared.

The preparation of a 90 per cent alcoholic extract as described is both laborious and costly. The labor and cost could be greatly reduced if the selective action exercised by 90 per cent alcohol were also exercised by 80 per cent alcohol. To determine this, extracts of rice bran and rice polish were prepared, using the same procedure adopted in the preparation of the 90 per cent alcoholic extracts and residues, the only difference being that the syrups obtained after distilling off the 25 per cent alcohol were brought to a concentration of 80 per cent alcohol instead of 90 per cent. The procedure was elaborated to the extent that an "activated" fuller's earth was prepared, and the vitamin B was dissolved from the earth with $Ba(OH)_2$ following the procedure of Jansen and Donath (8), which is essentially the same as that first proposed by Seidell (9). It was hoped to leave behind in these steps any of the residual heat-stable, water-soluble vitamin.

The "activated" fuller's earth was prepared as follows: One volume of rice bran extract as prepared by our 80 per cent alcoholic treatment (1cc. = 10 gm. of rice bran) was diluted with 3 volumes of water, and the pH roughly adjusted to 4.5 with the aid of the indicator, brom cresol green. It was shaken with fuller's earth in two portions, the total fuller's earth used being calculated to carry the equivalent of 40 gms. of rice bran (4 cc. of rice bran extract) for each gram of earth. Each portion of fuller's

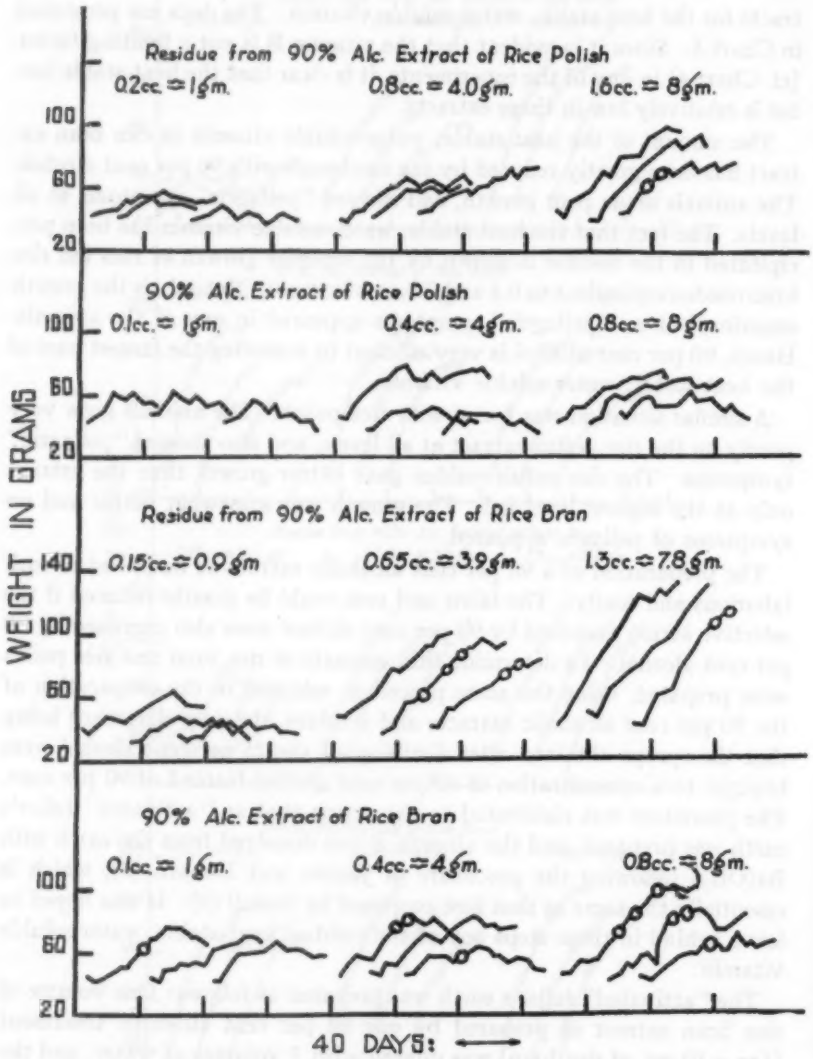
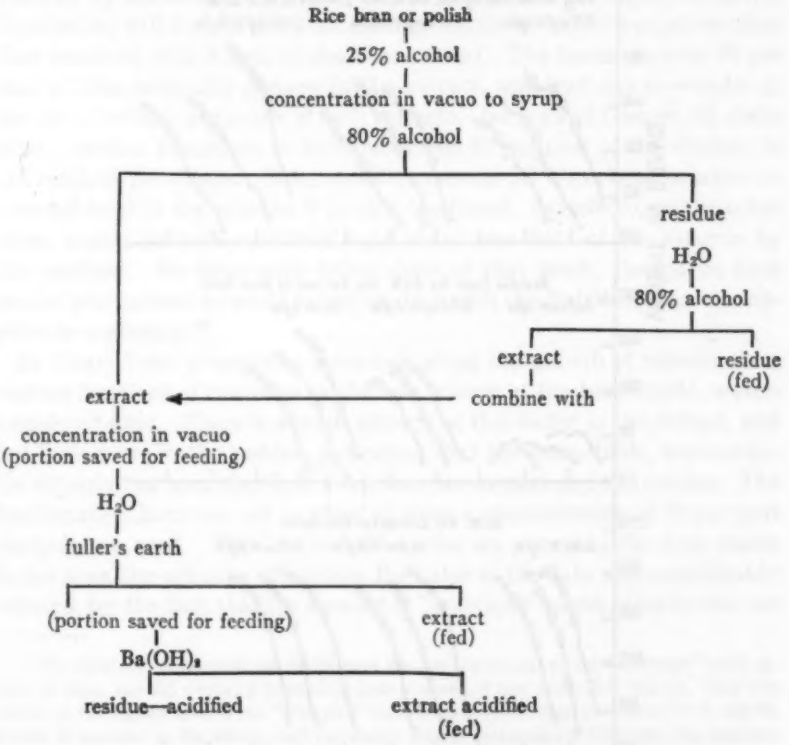


CHART 5.—Curves which demonstrate the amount of the heat-stable factor in a 90% alcoholic extract and residue prepared from a 25% alcoholic extract. Extracts of rice bran and rice polish were used. The animals were maintained on Diet 540 from the 21st to the 33rd day; at this time the extracts were started.

earth was filtered off on the Buchner funnel, washed with acidulated water, alcohol and ether. The two portions were then combined, mixed and fed to determine its vitamin content.

A portion of this "activated" fuller's earth was shaken up with a little water, and then $Ba(OH)_2$ solution was added until tropeolin O turned brown. It was thoroughly shaken, quickly filtered under suction and immediately acidified with 20 per cent H_2SO_4. This extraction was repeated twice. The combined extract was brought to neutrality with $Ba(OH)_2$ and then slightly acidified, filtered free of $BaSO_4$ and finally concentrated in vacuo so that 1 cc. of extract was equivalent to 48 gms. of bran. Following is a diagramatic outline of the procedure.



In Chart 6 are presented curves which show the amount of vitamin B in the various rice bran fractions. As is to be expected, the extracts are very potent in vitamin B, 0.1 cc. fully satisfying the vitamin B requirements of

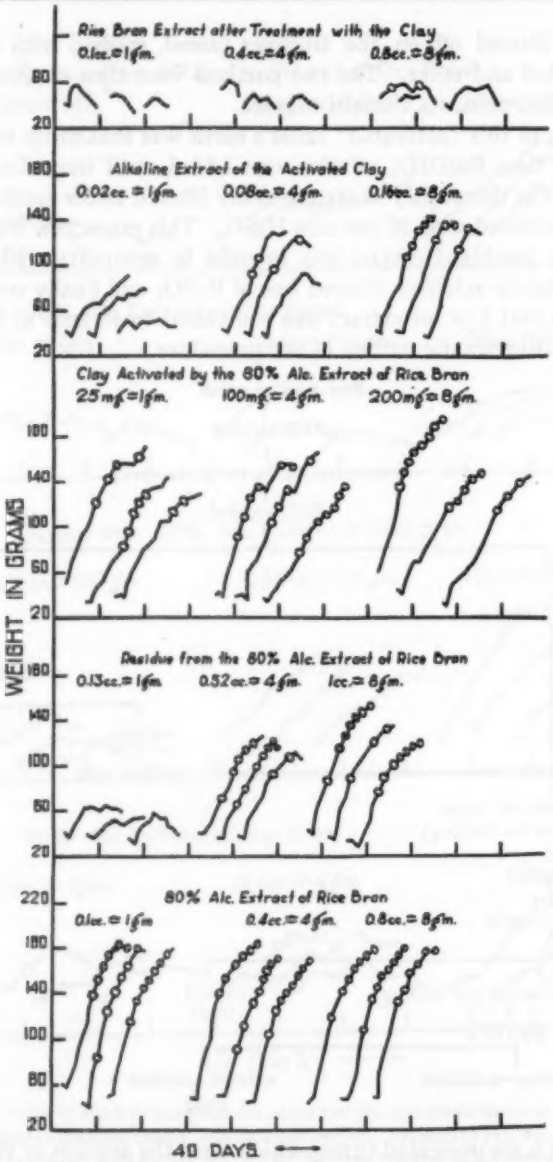

Rice Bran Extract after Treatment with the Clay
0.1cc.=1gm. 0.4cc.=4gm. 0.8cc.=8gm.
Alkaline Extract of the Activated Clay
0.02cc.=1gm. 0.08cc.=4gm. 0.16cc.=8gm.
Clay Activated by the 80% Alc. Extract of Rice Bran
25mg.=1gm. 100mg.=4gm. 200mg.=8gm.
Residue from the 80% Alc. Extract of Rice Bran
0.13cc.=1gm. 0.52cc.=4gm. 1cc.=8gm.
80% Alc. Extract of Rice Bran
0.1cc.=1gm 0.4cc.=4gm. 0.8cc.=8gm.
WEIGHT IN GRAMS
40 DAYS

CHART 6.—Curves which demonstrate the amount of vitamin B in 1. an 80% alcoholic extract prepared from a 25% alcoholic extract of rice bran, 2. the residue, 3. a clay activated by the extract, 4. an alkaline extract of the activated clay and 5. the extract after treatment with the clay. The animals received Diet 540 from the 21st to the 33rd day. At this time Diet 542 replaced Diet 540 and the extracts were fed.

the rat. On the other hand, the potency of the residue has been greatly reduced; the highest level fed did not give the growth obtained by 0.1 cc. of the extract. We had difficulty in determining the vitamin content of the "activated" fuller's earth since the rats receiving the equivalent of 0.1 cc. of extract were the only ones to eat all the "activated" earth offered to them. As can be seen, excellent growth was obtained on this lowest level of fuller's earth.*

That the fuller's earth had removed practically all the vitamin B from solution is confirmed by the lack of response of the animals when fed the rice bran extract after it had been shaken with fuller's earth. Failure in growth resulted at all levels of this extract. The extract obtained from the clay by use of $Ba(OH)_2$ suffered great losses. On the highest level fed, (equivalent to 0.8 cc. of rice bran extract) the performance was poorer than that obtained with 0.1 cc. of rice bran extract. The losses are over 90 per cent of that originally present in the extract, and lead one to wonder at the practicability of the use of such extracts. Jansen and Donath (8) claim after a similar procedure to have recovered 80 per cent of the vitamin in the original polishings. Guha and Drummond (4) also claim to have recovered most of the vitamin B by this treatment. Seidell (9), on the other hand, claims to have recovered only about one-third of the vitamin by this method. We have even fallen short of that mark, though we took special precautions to avoid longer contact with the $Ba(OH)_2$ than was absolutely necessary.**

In Chart 7 are presented curves indicating the growth of rats on these various fractions of rice bran as the sole source of the heat-stable, water-soluble vitamin. There is a small amount of this factor in the extract, and but little more in the residue, indicating that the heat-stable, water-soluble vitamin has been distributed between the extract and the residue. The fractionation here was not as great as when a concentration of 90 per cent alcohol was used. The "activated" earths are poorer in the heat stable factor than the extracts. Here too, the value of the data was considerably reduced by the fact that the amount of "activated" earth eaten by the rat

* The other curves are quite unreliable since the rats did not eat all the "activated" earth offered to them; nor did we find it possible to keep a record of how much they did eat. Only one animal on the highest level of the "activated" fuller's earth ate all that was offered to it, and its growth is superior to the others, and represents normal performance. However, the superior performance of the animal must be viewed with caution, since it was an unusual animal, weighing more than most of the animals under the same conditions.

** It is possible that these discrepancies are due to differences in technique. For example, we maintain animals over a longer test period, and on a diet most sensitive to vitamin B deficiency.

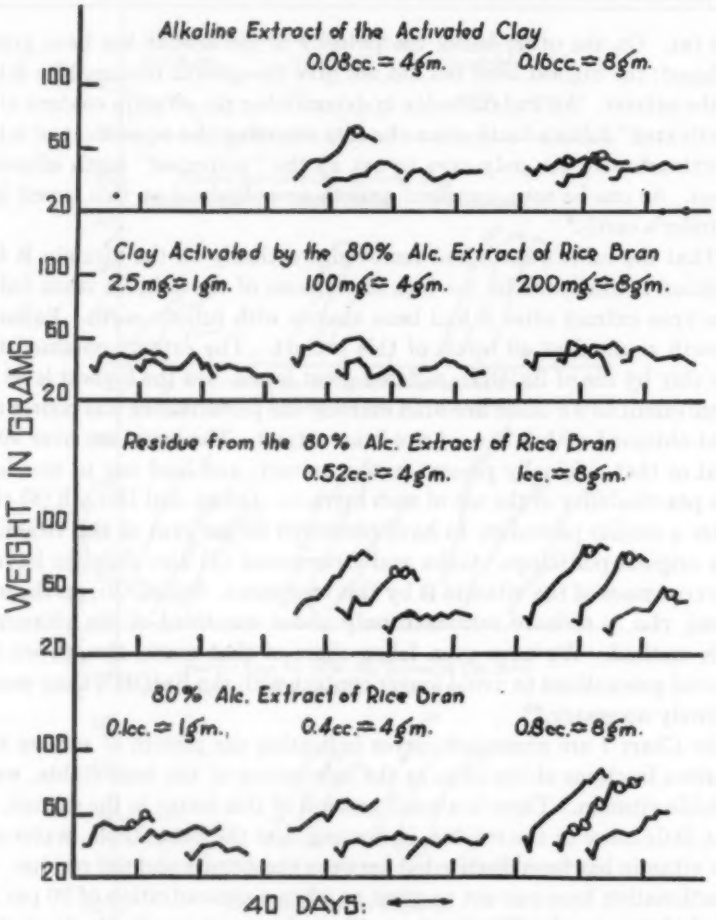


CHART 7.—These curves indicate the amount of the heat-stable factor in 1. an 80% alcoholic extract prepared from a 25% alcoholic extract of rice bran, 2. the residue, 3. a clay activated by this extract and 4. an alkaline extract of the activated clay. The animals were maintained on Diet 540 from the 21st to the 33rd day, then the extracts were fed.

was unknown. From these and other studies to be reported in a later section of this paper, we feel that "activated" fuller's earth should be an excellent source of vitamin B when the presence of a minimum amount of the heat-stable water-soluble vitamin is desired.*

* It should be recalled that Funk and Dubin (10) in 1921 prepared an activated fuller's earth from yeast extract that did not support growth in rats. They recognized then that two vitamins of

This whole procedure was repeated with rice polish, with the following addition, namely: the acidification of the fuller's earth after treatment with $Ba(OH)_2$ washing, drying and feeding to determine if any residual vitamin B remained unextracted in the fuller's earth.

The results are presented in Chart 8. Here as with rice bran extract, 0.1 cc. of rice polish extract gave maximum growth. The extraction was quite complete and therefore losses in the residue were greatly reduced. The clay carrying the equivalent of 0.1 cc. of the rice polish extract is slightly inferior to the untreated extract. We wonder whether this actually represents a decrease in vitamin B. The uniform failure of the animals on the rice polish extract after it had been shaken with fuller's earth strongly suggests that *all* the vitamin B has been adsorbed by the fuller's earth.

To feed higher levels of "activated" fuller's earth, we of course gave up our previous attempts to feed it separately and mixed it in the diet. Consumption records were kept in order to determine the equivalent of rice extract the rats consumed. The "activated" fuller's earth was fed at the levels of 2 per cent and 4 per cent. The rats on the 2 per cent level consumed fuller's earth approximately equivalent to 0.8 cc. of rice polish extract. The growth, however, is no better than that on the equivalent of 0.1 cc. of rice polish extract. We cannot explain it, but it is unlikely that it is due to deficiency in vitamin B. The rats on the 4 per cent level of fuller's earth ate the equivalent of 2.2 cc. of rice polish extract, an amount greatly in excess of any level of rice extract fed. The growth curves of these animals are superior to any others in this series.

The extract obtained from the "activated" fuller's earth with $Ba(OH)_2$ again showed tremendous losses in vitamin B. At the highest level the growth was somewhat inferior to the lowest level on the rice polish extract so that the recovery is of the order of 10 per cent. We examined the residual clay and found it very poor in vitamin B so we are forced to conclude that our losses approximating 90 per cent are due to actual destruction or inactivation in some other way.

The amount of the heat-stable, water-soluble vitamin in the various rice polish fractions is shown by the curves in Chart 9. Since the original rice polish was poor in the heat-stable factor, there was little opportunity for much fractionation. The animals receiving the fuller's earth were poorer than those receiving the extract. The animals on the highest level consumed "activated" earth carrying the equivalent of about 0.8 cc. of

the B type were necessary for growth and well being. Williams and Waterman (11) have recently reported similar experiments.

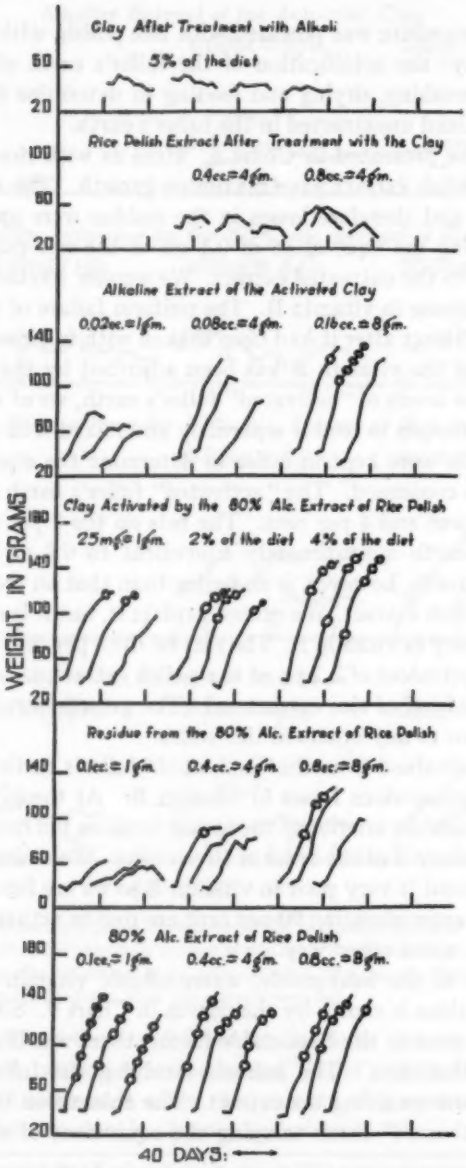

Clay After Treatment with Alkali
5% of the diet
Rice Polish Extract After Treatment with the Clay
0.4cc.=4gm. 0.8cc.=4gm.
Alkaline Extract of the Activated Clay
0.02cc.=1gm. 0.08cc.=4gm. 0.16cc.=8gm.
Clay Activated by the 80% Alc. Extract of Rice Polish
25mg.=1gm. 2% of the diet 4% of the diet
Residue from the 80% Alc. Extract of Rice Polish
0.1cc.=1gm. 0.4cc.=4gm. 0.8cc.=8gm.
80% Alc. Extract of Rice Polish
0.1cc.=1gm. 0.4cc.=4gm. 0.8cc.=8gm.
WEIGHT IN GRAMS
40 DAYS

CHART 8.—Growth curves which demonstrate the amount of vitamin B in 1. an 80% alcoholic extract prepared from a 25% alcoholic extract of rice polish, 2. the residue, 3. a clay activated by this extract, 4. an alkaline extract of the activated clay, 5. the extract after treatment with the clay (This portion of chart should read 0.8 cc. = 8 gm instead of 4 gm.) and 6. the clay after treatment with alkali ($Ba(OH)_2$). The animals received Diet 540 from the 21st day to the 30th day. Then Diet 542 replaced Diet 540 and the extracts were fed.

rice polish extract. We are certain that vitamin B was not a limiting factor, and therefore it is reasonable to conclude that the small amount of the heat-stable factor originally present in the 25 per cent alcoholic extract of rice polish had been largely removed in the various manipulations

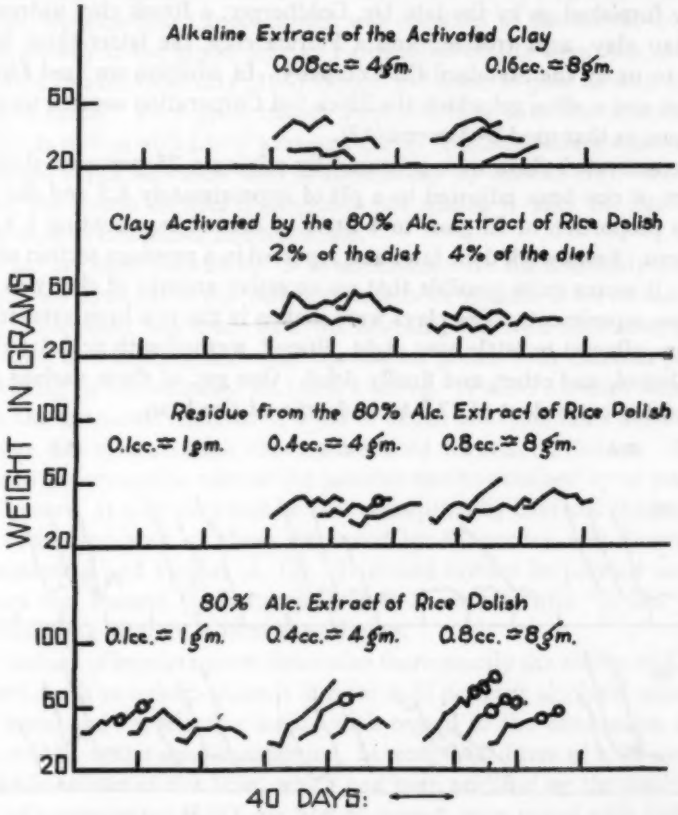


CHART 9.—These curves indicate the amount of the heat-stable factor in 1. an 80% alcoholic extract prepared from a 25% alcoholic extract of rice polish, 2. the residue, 3. a clay activated by this extract, and 4. an alkaline extract of the activated clay. The animals received Diet 540 from the 21st to the 30th day and then the fractions were fed.

necessary to obtain the "activated" fuller's earth. Since the fuller's earth is practically devoid of the heat-stable, water-soluble vitamin, it is not surprising that we see no evidence of it in the extract obtained from the "activated" earth.

Adsorption Studies with Silicious Materials

In our attempts to prepare a concentrated vitamin B preparation as free as possible from impurities, we tested out the ability of various clays and charcoals to adsorb vitamin B. We used an English fuller's earth (Surrey) kindly furnished us by the late Dr. Goldberger; a Brock clay untreated; an Otay clay, acid treated; and a Florida clay, the latter three being given to us by the Standard Oil Company. In addition we used Lloyd's reagent and a silica gel which the Silica Gel Corporation assured us to be the same as that used by Levene (12).

The activated clays were prepared by mixing a 25 per cent alcoholic extract of rice bran adjusted to a pH of approximately 4.5 and the clay in the proportion of 75 gms. to 4 liters of extract representing 1 kg. of rice bran. From work done later and reported in a previous section of this paper, it seems quite possible that an excessive amount of clay was used in these experiments. The clays were shaken in the rice bran extract 2 to 3 hours, allowed to settle over night, filtered, washed with acidulated water, alcohol, and ether, and finally dried. One gm. of these various clays carried the equivalent of 12.0 to 12.4 gms. of rice bran.

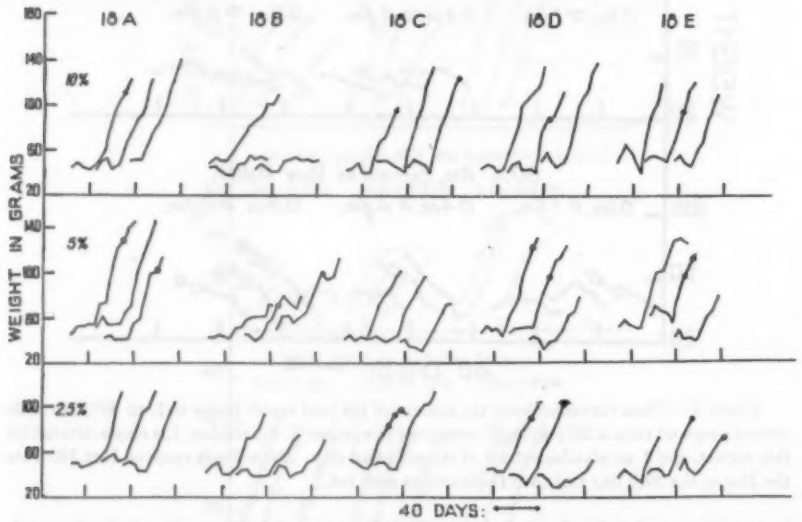


Chart 10.—Curves which demonstrate the absorptive action of various clays for vitamin B in a 25% alcoholic extract of rice bran. The clays were fed as 2.5%, 5% and 10% of the diet (542). The animals were maintained on Diet 540 from the 21st to the 37th day. At this time Diet 542 replaced Diet 540. The various clays were added on the 45th day: 18A, English fuller's earth; 18B, the untreated Brock clay; 18C, the Otay clay; 18D, Florida clay; 18E, Lloyd's reagent.

The ability of these various clays to adsorb vitamin B is demonstrated by the curves presented in Chart 10. The clays were mixed in the diets at 2.5 per cent, 5 per cent and 10 per cent levels, replacing an equivalent amount of diet. Excellent results were obtained with the English fuller's earth (18A). The untreated Brock clay (18B) apparently exerted a toxic action, because fairly good growth was obtained at the lowest level and poor growth at the higher levels. Fairly good results were obtained with the acid treated Otay clay (18C) though not quite so good as with English fuller's earth. Very good results were obtained with the Florida clay, (18D) as well as with Lloyd's reagent (18E). Uniform failure was met with silica gel which had been used so successfully by Levene (12). We do not know the reason for this discrepancy but realize it may be due to the difference in conditions and materials.

What are most difficult for us to understand are the uniformly negative results we obtained in our attempts to adsorb vitamin B on animal charcoal. One effort was made near the neutral point with untreated animal charcoal, and another, also near the neutral point, with charcoal purified by boiling in concentrated HCl and washing. A third attempt was made with the untreated charcoal at a pH of about 4.5. The three attempts to remove the vitamin with charcoal resulted in uniform failure. This is hard to understand in view of the positive results obtained by so many investigators. It may very well be that adsorption by charcoal requires special conditions such as those employed by Kinnersley and Peters, and Chamberlain and Vedder (3, 13). It should further be pointed out that Jansen and Donath (8) decolorized their B extracts with "norite" without removing vitamin B from the solution.

It seemed of importance to determine more exactly the ability of English fuller's earth to adsorb vitamin B from a 25 per cent alcoholic extract of rice bran, and its selective action with regard to the elimination of the heat-stable, water-soluble vitamin. Seventy-two liters of a 25 per cent alcoholic extract of rice bran, which had been acidified by the addition of 3 cc. of concentrated H_2SO_4 per liter of extract, were mixed with 1600 gm. of fuller's earth, and the mixture shaken repeatedly for one and one-half days.* Four liters of the extract carried the equivalent of 1 kg. of rice bran. The clay was washed with 25 per cent acidulated alcohol and then with ether. One gm. of clay, 23-K, carried the equivalent of approximately 11 gm. of rice bran.

* In this particular preparation the pH was 3.0 to 3.5 and the indicator used was brom phenol blue. Previous experiments showed that adsorption was readily accomplished under these conditions.

Chart 11 contains growth curves which illustrate the amount of vitamin B carried by this clay. Fifty mg. of clay carry the equivalent of 0.55 gm. of rice bran, and the growth on this compares favorably with the growth of rats on the original rice bran extracts. When fed 200 mgs. clay, the equivalent of about 2 gms. of rice bran, excellent growth was obtained. When 500 mgs. of the clay were fed, the animals did not eat all the clay offered to them. This necessitated the mixing of the clay in the food at a level of 10 per cent. The best performance was obtained at this level (5.5 gms. of rice bran), but it is not greatly superior to the performance obtained on the 200 mg. level. We believe we are justified in concluding that practically all of the vitamin B is adsorbed from the rice bran extract under the conditions of this experiment.

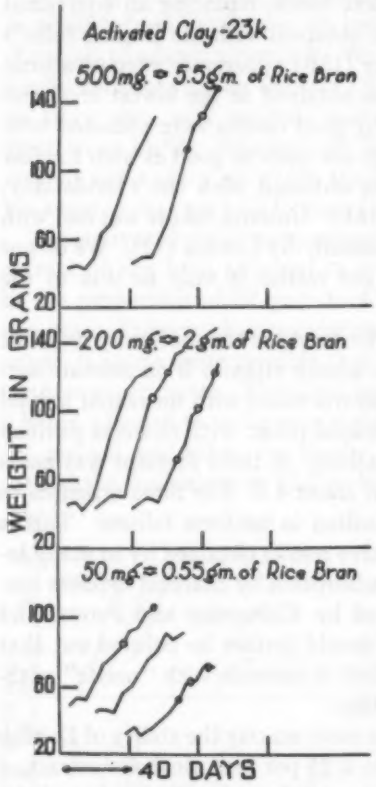


CHART 11.—Growth curves which indicate the amount of vitamin B removed by English fuller's earth (23-K) from a 25% alcoholic extract of rice bran. The treatment was more extensive than that with the same clay illustrated in Chart 10. The animals were on Diet 540 from the 21st to the 36th day. At this time Diet 542 replaced Diet 540 and the clay was fed at the levels indicated. (The 500 mg. level was fed as 10% of the diet after the 63d day.)

Having determined that vitamin B was not a limiting factor in the clay, we next fed it to determine its value in the heat-stable, water-soluble vitamin. The results are shown in Chart 12. Little or no growth took place, and symptoms of pellagra appeared in practically all the animals. This clay therefore furnishes a fine medium for the removal of vitamin B free of the heat-stable factor from a 25 per cent alcoholic extract of rice bran.

SUMMARY

To judge from our results with 25 per cent alcohol, an efficient extractive of both the heat-labile and heat-stable, water-soluble vitamins, the heat-stable factor is in low concentration in the outer layers of the rice

kernel and decreases further in abundance in the inner layers, whereas the heat-labile factor is in high concentration in the rice outer layers and decreases only slightly in the inner layers. Clay activated by the 25 per cent alcoholic extract furnishes an excellent source of vitamin B free from the heat-stable factor.

When the syrup resulting from concentration of a 25 per cent alcohol extract is treated with 95 per cent alcohol so that the resulting concentration is 90 per cent, most of the heat-stable factor is removed; nevertheless, large losses in the heat-labile factor are produced in this way. When the final concentration is 80 per cent these losses are much reduced, though unfortunately somewhat more heat-stable vitamin is present. In the case of the rice polish, so little of the heat-stable factor is initially present that fractionation with the 80 per cent alcohol yields an extract almost free of the heat-stable factor and very rich in vitamin B. Finally, adsorption with clay appears more selective for the heat-labile vitamin and thus the last traces of the heat-stable factor are left behind. Though we have not succeeded, as have some investigators, in approximating quantitative removal of anti-neuritic B from clays by means of barium hydroxide, the feeding of these clays has given us an adequate and, indeed, very high dosage of anti-neuritic B with assured absence of the heat-stable factor.

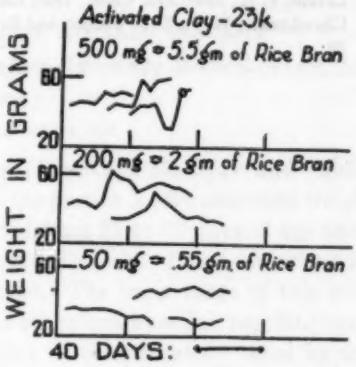


CHART 12.—Growth curves which indicate the amount of the heat-stable factor removed by English fuller's earth (23-K) from a 25% alcoholic extract of rice bran. The vitamin B needs were adequately satisfied as is shown in Chart 11. The animals were fed Diet 540 from the 21st to the 36th day. At this time the activated clay was fed at the levels indicated. (The 500 mg. level was fed as 10% of the diet after the 63d day.)

BIBLIOGRAPHY

1. Goldberger, J., G. A. Wheeler, R. D. Lillie, and L. M. Rogers, *U. S. Pub. Health Rep.*, 1926, XLI, 297.
2. Chick, H., and M. H. Roscoe, *Biochem. Jour.*, 1929, XXIII, 504.
3. Kinnersley, H. W., and R. A. Peters, *Biochem. Jour.*, 1927, XXI, 777.
4. Guha, B. C., and J. C. Drummond, *Biochem. Jour.*, 1929, XXIII, 880.
5. Reed, J. B., and F. W. Liepsner, *U. S. Dept. of Agric.*, Bull. 570, 1917.
6. Evans, H. M., and S. Lepkovsky, *This Journal*, 1929, II, 1.
7. Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists. The Association of Official Agricultural Chemists, Washington, D. C., 1920.

8. Jansen, B. C. D., and W. F. Donath, *Proc. K. Akad. Wetensch. Amsterdam*, 1926, XXIX, 1390.
9. Seidell, A., *Jour. Amer. Chem. Soc.*, 1922, XLIV, 2042.
10. Funk, C., and H. E. Dubin, *Proc. Soc. Exper. Biol. Med.*, 1921, XIX, 15.
11. Williams, R. R., and R. E. Waterman, *Proc. Soc. Exper. Biol. Med.*, 1927, XXV, 1.
12. Levene, P. A., *Jour. Biol. Chem.*, 1928, LXXIX, 465.
13. Chamberlain, W. P., E. B. Vedder, and R. R. Williams, *Philippine Jour. Sci.* (B) 1912, VII, 39.

FACTORS WHICH DETERMINE RENAL WEIGHT*
VII. PROTEIN INTAKE AND AGE.
(SECOND PAPER)

By

EATON M. MACKAY AND LOIS LOCKARD MACKAY

(From the Department of Medicine, Stanford University Medical School, San Francisco.)

Received for Publication—May 1, 1930

IN ANOTHER paper of this series (MacKay, MacKay and Addis, 1928a) a linear relationship between the protein intake and renal weight was demonstrated in young male rats fed from 26 to 70 days of age upon special diets, containing different amounts of casein. The summarized results of three experiments were presented. The importance of this relationship in connection with the study of other factors which regulate renal weight and its bearing upon the subject of compensatory renal hypertrophy—a study which will be considered elsewhere—has led us to collect further data in support of the earlier work. These are presented here in addition to similar details of the first experiments which are treated in a somewhat different manner than in the first report.

The methods which were used in the present study have already been described (MacKay and MacKay, 1927; MacKay, MacKay and Addis, 1928a). One important change in the treatment of the data should be noted. In the earlier experiments the body surface, which was used during the course of the experiment as a basis for measuring the food and protein intake, was determined from the mean gross body weight of each day. The kidney weight with which the protein intake was compared, on the other hand, was based upon the body surface at death determined from the gross body weight at death minus the contents of the gastro-intestinal tract, giving what we have called the "corrected body weight." In order to place the kidney weight and protein intake with which it is compared upon a more comparable basis, the body surfaces before death have been determined in the present instance from the mean gross body weight of each day minus a correction of 5 per cent for the probable weight of the intestinal contents. Another refinement in the calculation of the results reported here concerns the protein intake. Heretofore the average daily protein intake was determined by averaging the entire period of feeding the special

* This investigation was made possible by the Edward N. Gibbs Prize Fund of the New York Academy of Medicine.

diet, 44 days in the case of the young rats and 54 days for the year old groups. It has recently been found (Addis, 1929) that the renal hypertrophy which follows the administration of a high protein diet has almost reached completion within ten days after the diet has been commenced and without question a period of no more than twenty days is involved. Data obtained upon the former basis (an average of the entire experimental feeding period) led us to conclude (MacKay, MacKay and Addis, 1928b) that in male rats age has no influence upon the linear relationship between renal weight and the protein intake. The possibility of our being in error in this conclusion has led to a reinvestigation of the whole question of the effect of age on the relation of renal weight to the protein intake with the latter calculated from the ten-day period before death. Ten days were chosen rather than 20 because it is naturally desirable to select as short a period as is feasible before the anatomical measurements are made.

Seven diets, each containing a different protein concentration, were used. The ingredients and composition of these are given in Table I. The protein

TABLE I

Ingredient	1	2	3	4	5	6	7
Casein	8.0	16.0	24.0	32.0	46.0	60.0	75.0
Cornstarch	52.0	45.0	37.0	29.0	20.0	11.0	4.0
Lard	15.0	15.0	15.0	15.0	10.0	5.0	0.0
Cod-liver oil	9.0	9.0	9.0	9.0	9.0	9.0	10.0
Salt mixture	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Dried yeast	9.0	9.0	9.0	9.0	9.0	9.0	9.0
Agar-agar	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Calories per 100 gm.	471	472	472	471	442	424	389
	Per cent of diet						
Protein	12.4	18.0	24.7	31.2	42.9	54.6	67.2
Fat	24.1	24.2	24.3	24.4	19.6	14.7	10.9
Carbohydrate	48.1	42.1	35.0	28.1	20.4	12.7	3.2
Water	5.5	5.6	5.7	5.8	6.3	6.8	7.1
Roughage	4.7	4.7	4.7	4.7	4.7	4.7	4.7
Salts	5.2	5.4	5.6	5.8	6.1	6.5	6.9
	Per cent of calories.						
Protein	10.5	15.7	21.4	27.3	39.8	52.8	70.7
Fat	47.7	47.7	48.0	48.2	41.3	35.0	26.0
Carbohydrate	41.8	36.6	30.6	24.5	18.9	12.2	3.3

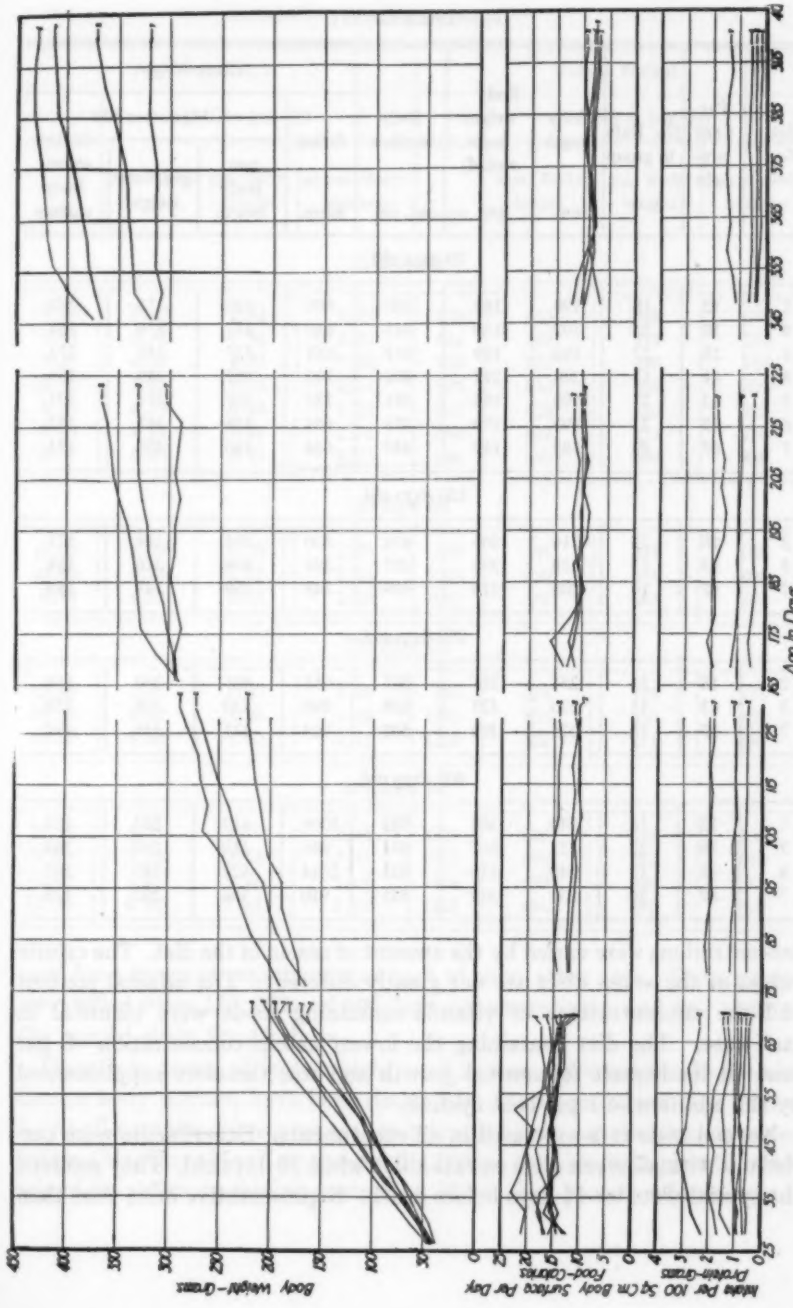

Body weight—Grams
Grams Per 100 Sq Cm Body Surface Per Day
Food—Calories
Protein—Grams
Age in Days

FIGURE 1.

TABLE II

Diet No.	Per Cent protein	No. Rats in group	Body length mm.	Body weight (corrected) gm.	Body surface sq. cm.	Heart Weight Actual mgm.	Heart Weight Mgm. per 100 mm. Body length	Heart Weight Mgm. per 100 gm. Body weight	Heart Weight Mgm. per 100 sq.cm. Body surface
70 days old									
1	12	18	190	163	338	608	320	373	180
2	18	25	192	170	348	640	333	376	184
3	25	22	193	189	373	651	337	345	175
4	31	18	204	212	403	740	362	349	183
5	43	21	198	195	381	730	369	374	191
6	55	22	193	189	373	684	354	362	183
7	67	29	189	159	332	644	340	405	194
130 days old									
2	18	25	214	266	471	830	384	314	177
5	43	15	219	283	487	895	408	316	184
7	67	16	208	218	409	748	359	343	183
220 days old									
2	18	24	231	361	567	941	407	260	164
5	43	16	230	325	536	990	430	304	185
7	67	16	229	294	502	964	421	328	192
400 days old									
1	12	14	240	382	582	1006	419	263	173
2	18	22	231	367	581	949	410	260	163
4	31	13	240	410	627	1014	422	247	161
7	67	26	231	312	521	910	394	292	175

concentrations were varied by the amount of casein in the diet. The caloric values of the seven diets are not greatly different. The mineral content and the concentrations of vitamin-containing foods were identical in each case. The diet containing the lowest casein concentration—8 per cent—is inadequate for normal growth and was therefore supplemented by the addition of 1 per cent cystine.

Normal male rats were used in all experiments. Experiments were carried out with all seven diets on rats killed when 70 days old. They received the special diets for 44 days before death. Representative diets were then

TABLE II (Continued)

Liver Weight				Kidney Weight			
Actual	Gm. per 100			Actual	Mgm. per 100		
gm.	mm. Body length	gm. Body weight	sq.cm. Body surface	mgm.	mm. Body length	gm. Body weight	sq.cm. Body surface
			70 days old				
9.68	5.10	5.94	2.87	633	333	388	187
8.42	4.38	4.94	2.42	644	335	379	185
8.27	4.28	4.37	2.22	738	382	390	198
10.30	5.05	4.86	2.54	836	410	394	207
8.69	4.39	4.45	2.28	832	420	426	218
9.40	4.87	4.97	2.52	894	463	473	239
9.07	4.80	5.70	2.73	964	510	606	289
			130 days old				
10.66	4.99	4.10	2.50	850	394	321	181
10.40	4.75	3.67	2.14	1005	459	355	206
8.58	4.12	3.93	2.10	1010	486	463	247
			220 days old				
12.60	5.45	3.49	2.19	986	428	274	172
11.57	5.03	3.56	2.16	1104	480	339	205
10.60	4.63	3.60	2.11	1210	528	411	240
			400 days old				
14.19	5.91	3.71	2.44	1024	427	268	166
12.89	5.54	3.51	2.21	1035	448	282	178
13.40	5.58	3.27	2.14	1102	459	269	176
11.36	4.92	3.64	2.16	1145	496	367	219

used for a group of experiments at each of three other ages. These groups were killed when 130, 220 and 400 days old respectively, and had received the special diets for 54 days.

Growth in terms of body weight, and food and protein intake in relation to body surface, have been charted for the 17 experiments in Figure 1. At all ages the differences are the same and bear out the idea expressed before (MacKay, MacKay and Addis, 1928a) that the best growth is obtained on a diet containing more than 30 per cent protein when casein is

the protein. An analysis of the rate of growth of the young rats on the various diets is presented in Figure 2. As the protein concentration is increased above 31 per cent, growth becomes progressively poorer. We have pointed out earlier that we believe this apparent inadequancy of the higher protein concentrations is due to the failure to maintain the ratio between

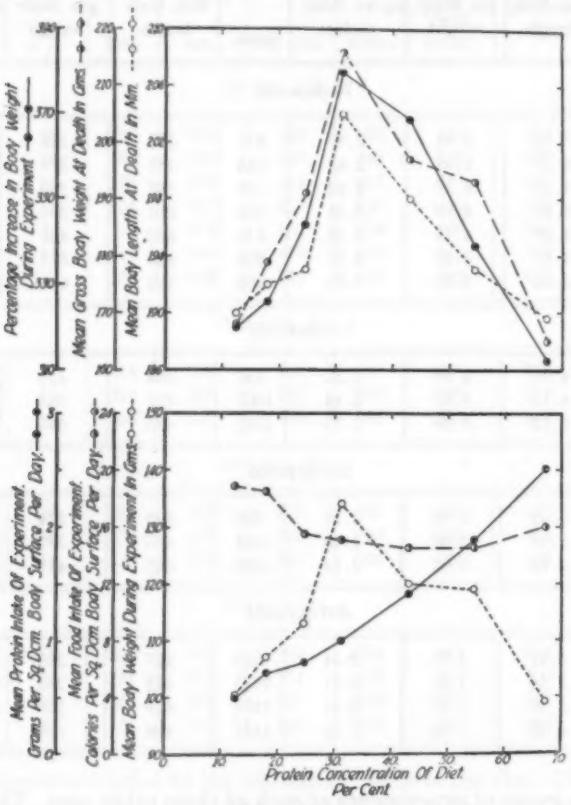

Percentage Increase In Body Weight During Experiment
Mean Gross Body Weight At Death In Gms.
Mean Body Length At Death In Mm.
Mean Protein Intake Of Experiment. Grams Per Sq. Dcm. Body Surface Per Day
Mean Food Intake Of Experiment. Calories Per Sq. Dcm. Body Surface Per Day
Mean Body Weight During Experiment In Gms.
Protein Concentration Of Diet Per Cent

FIGURE 2.

the protein and yeast extract contents of the diet which Reader and Drummond (1926) have found necessary. In other experiments we have found just as good growth on a 60 per cent protein diet as on a 30 per cent diet when the protein : yeast-extract ratio is maintained constant. Growth in both instances was appreciably better than when protein was 18 per cent of the diet.

The anatomical measurements made at death are summarized in Table II and the individual observations of kidney weight in relation to body surface charted in Figure 3. The mean renal weight figures at each age have been plotted against the protein intake in Figure 4 and fitted with the empirical formula, *protein intake* (grams per 100 sq. cm. body surface)

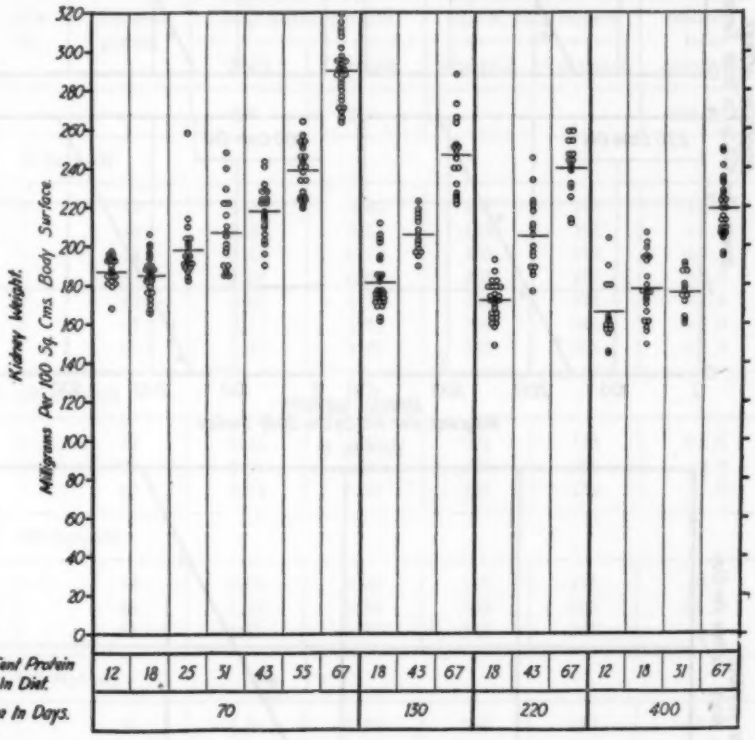


FIGURE 3.

*per day = 0.0183 renal weight** (milligrams per 100 sq. cm. body surface) -2.75, which was found best to represent the relationship for our earlier observations. Various other formulae of the same type were tried but none gave a better average result for all ages. In Table III are the average

* Throughout this series renal weight is given as the average weight of the two kidneys and consequently is half of the actual total.

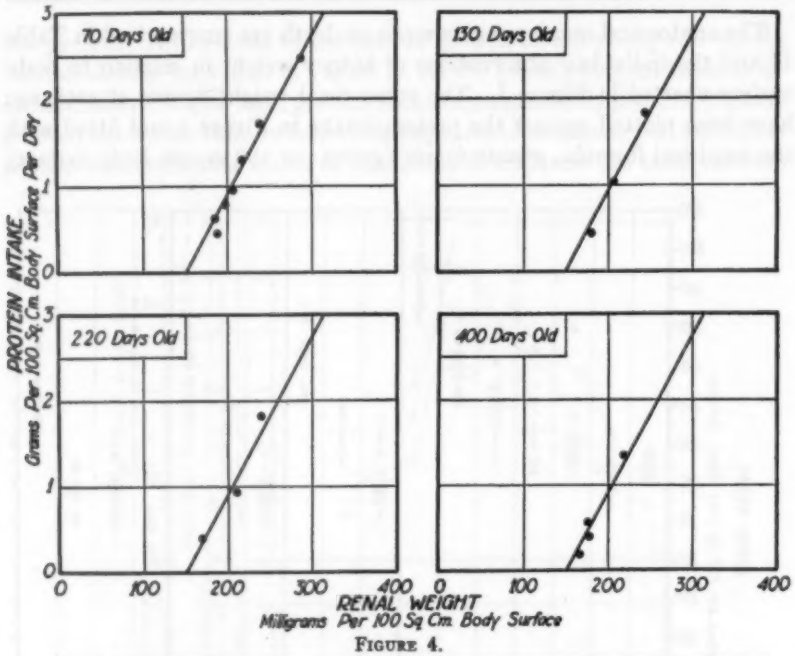


FIGURE 4.

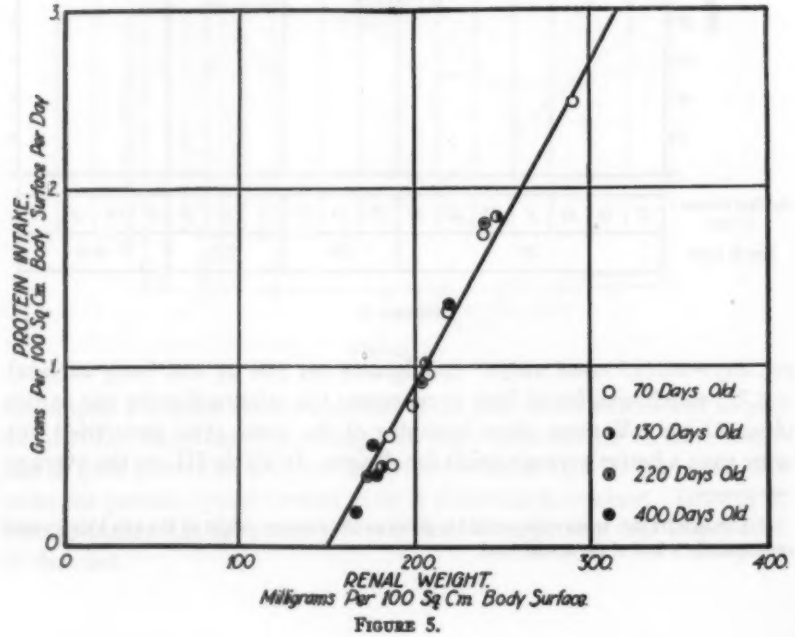


FIGURE 5.

figures and a comparison of the observed with the calculated kidney weights. Considering the nature of the data, the agreement is exceptionally good. In Figure 5 the mean observations at the four ages have been

TABLE III

Diet No.	Per cent protein	Intake per 100 sq. cm. body surface per day		Mgm. kidney per 100 sq. cm. body surface		Deviation of observed from calculated
		Food	Protein	Observed	Calculated	
		gm.	gm.			per cent
70 days old						
1	12	3.62	0.45	187	175	+6.9
2	18	3.39	0.61	185	183	+1.8
3	25	3.13	0.77	198	192	+3.1
4	31	3.04	0.95	207	202	+2.5
5	43	3.02	1.30	218	221	−1.4
6	55	3.15	1.72	239	244	−2.0
7	67	3.71	2.49	289	286	+1.5
130 days old						
2	18	2.41	0.43	181	174	+4.0
5	43	2.40	1.03	206	206	0.0
7	67	2.74	1.83	247	250	−1.2
220 days old						
2	18	2.07	0.37	172	171	−0.6
5	43	2.19	0.94	205	202	+1.5
7	67	2.68	1.79	240	247	−2.8
400 days old						
1	12	1.54	0.19	166	160	+3.7
2	18	2.11	0.38	178	171	+4.1
4	31	1.79	0.56	176	181	−2.8
7	67	1.99	1.34	219	223	−1.8

plotted together. There is no certain difference in the relationship with age. This confirms our earlier observations (MacKay, MacKay and Addis, 1928b).

In Figure 6 are plotted the protein intakes on the different diets for the purpose of demonstrating the effect of age on the protein intake with a

given diet. It is this variation of the food and protein intake with age which led us to suggest in a preliminary report (MacKay, MacKay and Addis, 1927) that the kidney weight was less influenced by the protein intake in old than in young rats. It happens of course (MacKay, MacKay and Addis, 1928b) that an increase of a given percentage in the protein

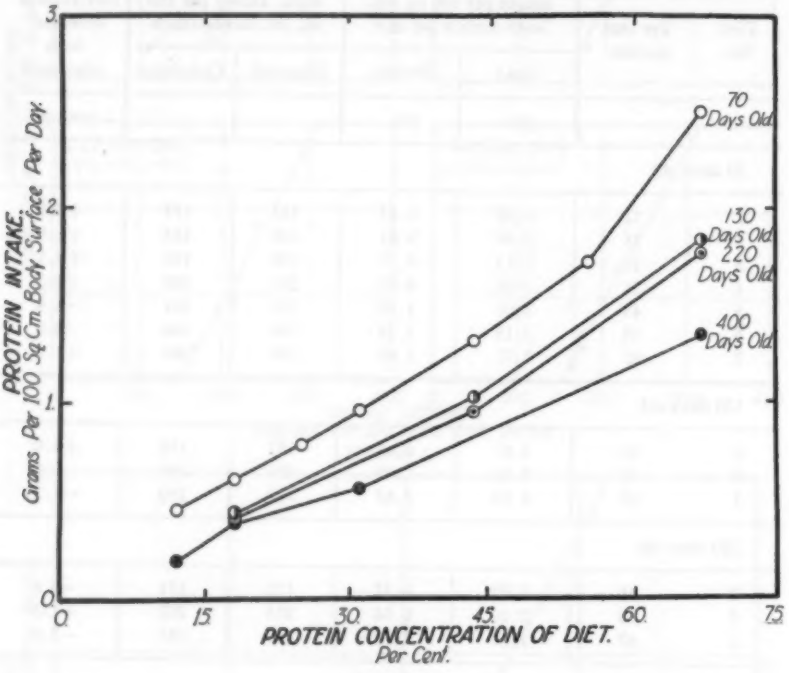


FIGURE 6.

intake will cause a larger percentage and actual increase in the renal weight of young rats because the protein intake per unit of body surface for a given diet is much lower in adult rats while renal weight as a function of body surface is essentially the same at all ages.

SUMMARY

An extended study of the linear relationship between renal weight and protein intake confirms our previous conclusion that it is uninfluenced by age. The empirical linear formula, *protein intake* $= 0.0183$ *renal weight* $- 2.75$, proved to be a satisfactory expression of the relationship in normal

male rats 70, 130, 220, and 400 days of age when receiving adequate amounts of protein in the form of casein.

REFERENCES

MacKay, E. M., L. L. MacKay, and T. Addis, 1928a, *Amer. Jour. Physiol.*, LXXXVI, 459; 1928b, *Ibid.*, 466.

MacKay, L. L., and E. M. MacKay, 1927, *Amer. Jour. Physiol.*, LXXXIII, 179.

Addis, T., 1929, Unpublished experiments.

Reader, V., and J. C. Drummond, 1926, *Biochem. Jour.*, XX, 1256.

MacKay, L. L., E. M. MacKay, and T. Addis, 1927, *Proc. Soc. Exp. Biol. and Med.*, XXIV, 335.

FACTORS WHICH DETERMINE RENAL WEIGHT*
VIII. PROTEIN INTAKE AND SEX

By

LOIS LOCKARD MACKAY AND EATON M. MACKAY

(From the Department of Medicine, Stanford University Medical School, San Francisco.)

Received for Publication—May 1, 1930

A LINEAR relationship between the protein intake, when in the form of casein, and kidney weight has been demonstrated (MacKay, MacKay and Addis, 1928a; 1928b; MacKay and MacKay, 1931) for male rats at all ages. The investigation presented here was designed to determine whether or not this relationship was affected by sex, that is, whether the same formula or one of a similar nature held for female rats. The experimental and other methods which were used have been detailed elsewhere (MacKay and MacKay, 1927a; 1928b; MacKay, MacKay and Addis, 1928a; 1928b; MacKay and MacKay, 1931). For purposes of comparison with the results on males, experiments were carried out with female rats upon three of the male diets. These rats were on the diets from 44 to 70 days of age. In a study of compensatory renal hypertrophy, to be reported elsewhere, all of our experiments on female rats were carried out with a female diet which differs considerably from the male diet (MacKay and MacKay, 1927a). Since we consider the ***renal weight : protein intake relationship*** of some importance in connection with compensatory renal hypertrophy, other experiments were also performed with variations of the female diet. These were carried out with each of three different diets on rats killed at 70, 130 and 220 days of age respectively. The youngest were on the special diet for 44 days and the rest for 54 days before being killed. In Table I the three special female diets are described. The differences in growth on the various diets as charted in Figure 1 are very similar to those of the male rats.

The average results of the anatomical measurements made at death are recorded in Table II and the individual observations of kidney weight in relation to body surface charted in Figure 2. The mean daily protein intake was calculated for the ten-day period preceding the termination of

* This investigation was made possible by the Edward N. Gibbs Prize Fund of the New York Academy of Medicine.

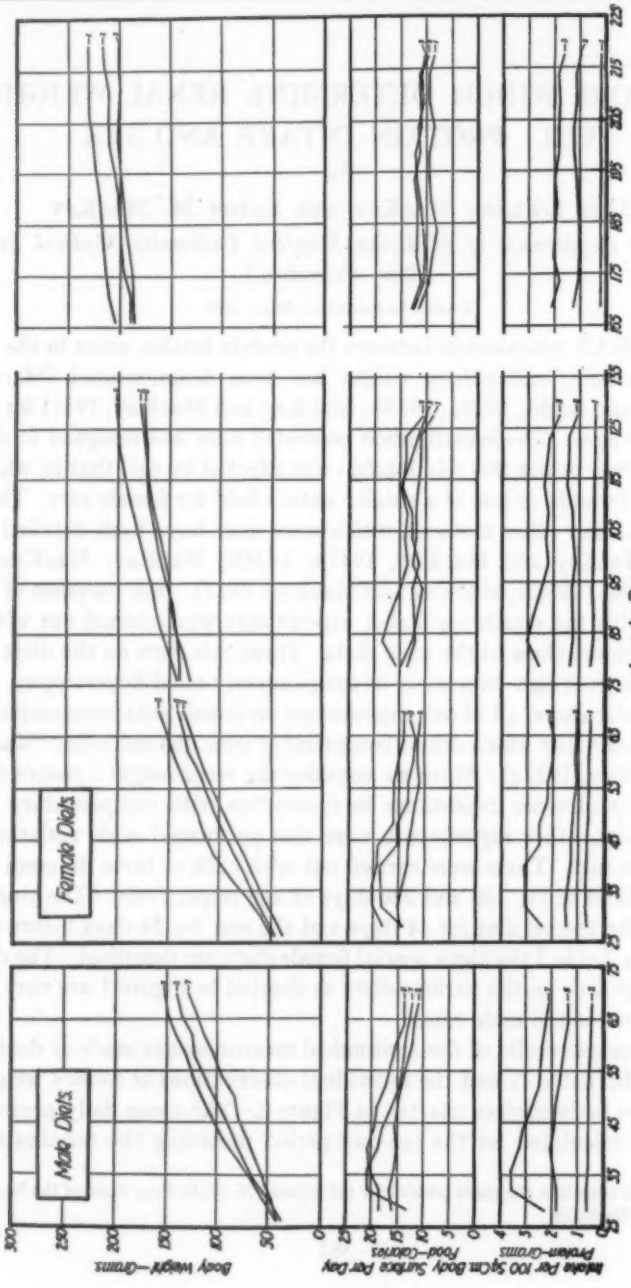

Male Diets
Female Diets
Body weight—Grams.
Intake Per 100 Sq.cm Body Surface Per Day
Food—Calories.
Protein—Grams.
Age in Days.

FIGURE 1.

TABLE I

Ingredient	Diet		
	1	2	3
Casein	15.0	50.0	71.0
Cornstarch	41.0	6.0	0.0
Lard	15.0	15.0	0.0
Cod-liver oil	9.0	9.0	9.0
Salt mixture	4.0	4.0	4.0
Dried yeast	4.0	4.0	4.0
Wheat germ	10.0	10.0	10.0
Agar-agar	2.0	2.0	2.0
Calories per 100 grams	479	470	389
	Per cent of Diet		
Protein	17.2	46.4	63.8
Fat	25.1	25.6	10.9
Carbohydrate	42.7	10.3	6.7
Water	6.3	8.2	8.5
Roughage	3.4	3.4	3.4
Salts	5.2	6.1	6.7
	Per cent of Calories		
Protein	14.8	40.5	67.1
Fat	48.6	50.5	25.9
Carbohydrate	36.6	9.0	7.0

each experiment by the method described in the preceding paper. In Table III are these average figures for protein consumption. These have been plotted in Figure 3 against the average kidney weight of each group. The line representing the empirical linear formula, *protein intake* $= 0.0183$ *renal weight** $- 2.75$, which was found best to express the relationship for male rats, has been drawn in for each age on the chart. It is obvious that this formula is not a satisfactory expression of the relation for female rats at any age. The observed renal weight is significantly lower in all groups than that (Table III) calculated by the male formula. The results in Figure 3 suggest no obvious difference between the protein intake and renal weight relationship on the male and female diets but the data are insufficient to

* Renal weight in this paper refers to the average weight of the two kidneys.

TABLE II

Diet No.	Per Cent protein	No. Rats in group	Body length mm.	Body weight (corrected) gm.	Body surface sq.cm.	Heart Weight Actual mgm.	Heart Weight Mgm. per 100 mm. Body length	Heart Weight Mgm. per 100 gm. Body weight	Heart Weight Mgm. per 100 sq.cm. Body surface
70 days old (male diets)									
2	18	22	177	135	298	491	277	363	165
5	43	22	185	148	317	579	313	391	183
7	67	22	172	124	281	502	292	404	187
70 days old (female diets)									
1	16	25	177	130	292	501	283	386	173
2	50	19	189	157	330	584	309	372	177
3	72	20	181	132	294	535	295	405	182
130 days old (female diets)									
1	16	28	190	173	352	560	295	324	159
2	50	16	200	200	387	701	350	350	181
3	72	15	194	170	348	618	318	363	178
220 days old (female diets)									
1	16	19	204	226	421	654	321	289	156
2	50	15	205	212	403	685	334	323	170
3	72	16	201	196	383	674	335	344	176

permit of any definite conclusion. They have accordingly been treated as a whole. A large number of formulae of different types and with various constants were tried out but none fits the observations at all ages any better than that of the line drawn in Figures 3 and 4. This has the formula: *protein intake = 0.0183 renal weight − 2.2875*. The maximum deviation of any observed value from the figure calculated by this expression is 6.8 per cent and the mean deviation of the observed from the calculated kidney weights only 2.9 per cent. This female formula is similar to the formula expressing the same relationship for male rats in that each increment in protein intake gives the same increase in renal weight. It differs only in that at a zero protein intake a theoretical renal weight of 125 milligrams

TABLE II (Continued)

Liver Weight				Kidney Weight			
Actual	Gm. per 100			Actual	Mgm. per 100		
gm.	mm. Body length	gm. Body weight	sq. cm. Body surface	mgm.	mm. Body length	gm. Body weight	sq. cm. Body surface
70 days old (male diets)							
5.58	3.15	4.13	1.87	482	272	357	162
6.80	3.67	4.59	2.14	643	347	434	202
6.12	3.55	4.93	2.18	680	395	548	235
70 days old (female diets)							
6.11	3.45	4.70	2.11	447	252	346	154
7.23	3.82	4.62	2.19	660	349	420	200
6.11	3.37	4.63	2.08	670	370	228	228
130 days old (female diets)							
6.63	3.49	3.83	1.88	510	269	296	145
7.78	3.89	3.89	2.01	726	363	363	188
6.96	3.59	4.09	2.00	736	379	433	212
130 days old (female diets)							
7.32	3.59	3.24	1.74	606	282	270	144
7.74	3.78	3.65	1.92	706	344	333	175
7.24	3.60	3.69	1.89	812	404	414	213

per 100 square centimeters body surface is demanded for female rats instead of the 150 milligrams per 100 square centimeters body surface which is indicated (MacKay, MacKay and Addis, 1928a) by the male formula. The difference between the two, or 25 milligrams, is a figure which coincides remarkably well with another sex difference. It was found (MacKay and MacKay, 1927b, 1927c) that the renal weight of 382 female rats of all ages was 154 milligrams and of 548 male rats of all ages 181 milligrams per 100 square centimeters body surface. The females then have 27 milligrams less of renal tissue per unit body surface. The rats of both of these groups were raised on diets of very similar protein content and caloric value.

Summary

A linear relationship between renal weight and protein intake has been demonstrated for female rats at all ages. When receiving adequate amounts of protein in the form of casein it may be expressed: *protein intake = 0.0183 renal weight −2.2875.* This is similar to the formula

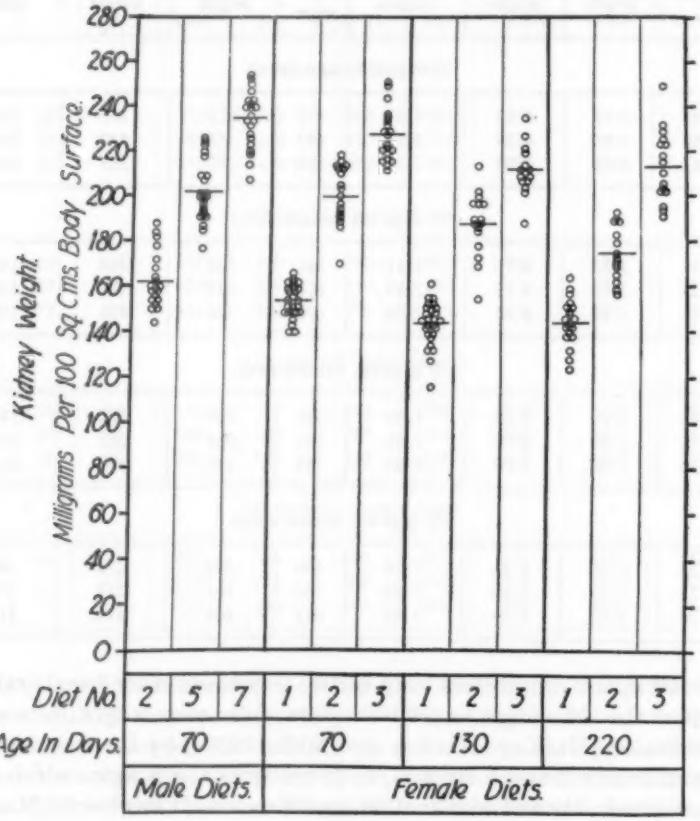


Figure 2.

expressing the same relationship for male rats, except that at a zero protein intake it demands a theoretical renal weight in relation to a unit of body surface (100 sq. cm.) of 125 instead of 150 milligrams, or 25 milligrams less.

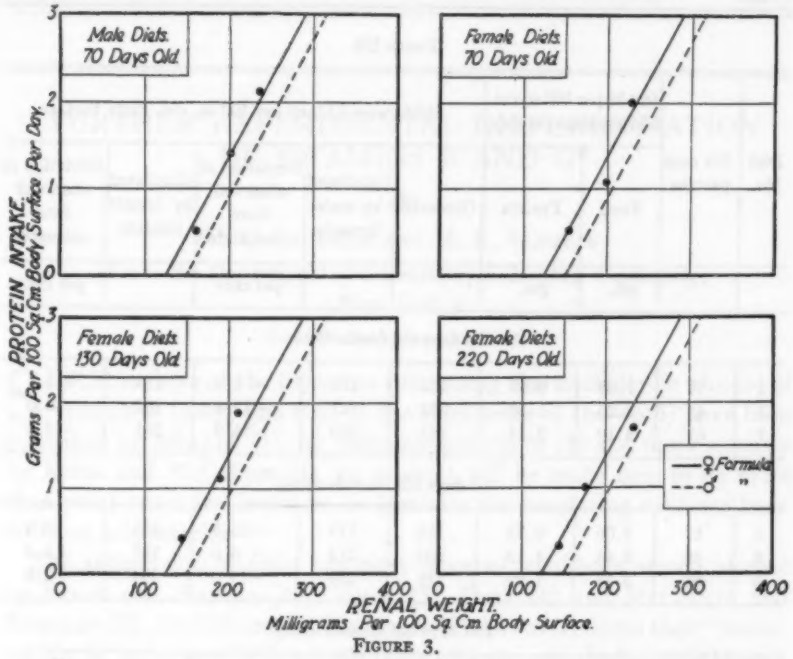


FIGURE 3.

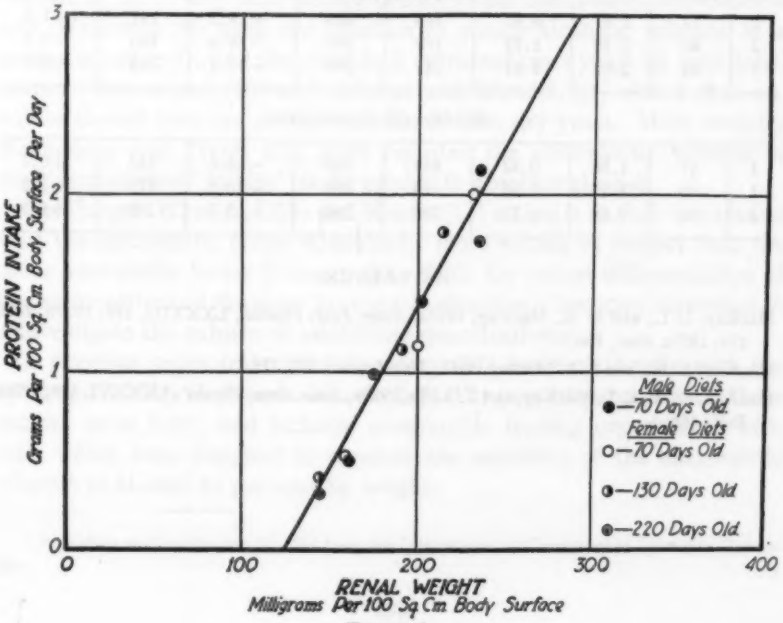


FIGURE 4.

TABLE III

Diet No.	Per cent protein	Intake per 100 sq.cm. body surface per day		Milligrams Kidney per 100 sq. cm. Body Surface				
		Food	Protein	Observed	Calculated by male formula	Deviation of observed from calculated	Calculated by female formula	Deviation of observed from calcuated
		gm.	gm.			per cent		per cent
70 days old (male diets)								
2	18	2.68	0.49	162	170	− 4.9	153	+5.5
5	43	3.26	1.40	202	227	−12.4	202	0.0
7	67	3.19	2.13	235	270	−14.9	241	−2.6
70 days old (female diets)								
1	17	3.06	0.53	154	179	−16.2	154	0.0
2	46	2.44	1.13	200	212	− 6.0	187	+6.5
3	64	3.13	1.99	228	259	−13.6	234	−2.6
130 days old (female diets)								
1	17	2.37	0.41	145	173	−19.3	147	−1.4
2	46	2.42	1.12	188	208	−10.6	186	+1.1
3	64	2.84	1.81	212	249	−17.4	244	−5.7
220 days old (female diets)								
1	17	1.88	0.32	144	168	−16.6	143	+0.7
2	46	2.12	0.98	175	204	−16.6	179	−2.3
3	64	2.68	1.71	235	244	− 3.8	219	+6.8

REFERENCES

MacKay, L. L., and E. M. MacKay, 1927a, *Amer. Jour. Physiol.*, LXXXIII, 179; 1927b, *Ibid.*, 191; 1927c, *Ibid.*, 196.

MacKay, E. M., and L. L. Mackay, 1931, *This Journal*, III, 375.

MacKay, E. M., L. L. MacKay, and T. Addis, 1928a, *Amer. Jour. Physiol.*, LXXXVI, 459; 1928b, *Ibid.*, 466.

FURTHER EXPERIMENTAL DIFFERENTIATION OF VITAMINS B AND G*

By

H. C. SHERMAN AND M. R. SANDELS

(From the Department of Chemistry, Columbia University, New York.)

Received for Publication—May 21, 1930

SINCE reviews of the literature concerning the solubility in alcohol of vitamin B (using this term in the older sense of the word) have been published by Mitchell (1), by Sherman and Smith (2) and more recently by Kruse and McCollum (3), no attempt will be made here to do more than point out a few instances to illustrate the conflicting evidence from different laboratories.

The antineuritic vitamin has been reported soluble in strong alcohol by Fraser and Stanton (4), Cooper (5), Funk (6) and McCollum and Kennedy (7). McCollum and Simmonds (8), however, found their "water-soluble B" to be very little soluble in cold 95 per cent alcohol and Osborne and Wakeman (9) used precipitation in strong alcoholic solution as a means of concentrating the vitamin B extracted from yeast by acidulated water. Drummond (10) and Osborne and Mendel (11) stated that absolute alcohol does not extract vitamin B from dry yeast. More recently Kinnersley and Peters (12) have reported the antineuritic vitamin of their concentrate "torulin" to be soluble in absolute alcohol.

Goldberger, Wheeler, Lillie and Rogers (13) stated it as their impression that the antineuritic factor is distinctly more soluble in alcohol than the more heat-stable factor (vitamin G). With the recent differentiation of the antineuritic and the more heat-stable vitamins it becomes important to reinvestigate the subject of solubilities quantitatively.

A previous paper from this laboratory (14) has dealt briefly with the sparing solubility of vitamin G in alcohol. The present paper reports these studies more fully, and includes comparable feeding experiments with rats, which were designed to measure the solubility of the antineuritic vitamin in alcohol 80 per cent by weight.

* Published as Contribution No. 644 from the Department of Chemistry of Columbia University.

Method of Extracting Yeast with Alcohol

The essential steps in the extraction experiments here described were as follows: 400 grams of air-dry bakers' yeast were treated with 1500 cc. of alcohol, thoroughly stirred and allowed to stand at room temperature (20°–25° C.) for 24 hours; then filtered with suction and the yeast washed on a Büchner filter with 750 cc. of alcohol of the same strength; then again stirred with 1500 cc. of the alcohol, allowed to stand 24 hours, filtered and washed as before. The residue was dried in the air at room temperature. The extract obtained by combining the two filtrates and the washings, when 80 per cent (by weight) alcohol was used, was a clear yellow solution of pH 6.1. It was concentrated on the steam bath and evaporated at room temperature on cornstarch. The dried product was ground and enough cornstarch added to make the final weight of the preparation 200 grams, so that each gram of the "activated" starch represented the alcoholic extract of two grams of dry bakers' yeast.

Solubility of Vitamin B

Experimental Methods. Healthy young rats of known nutritional history were put at 28 to 29 days of age upon the basal diet described below. One animal in each litter was kept upon the basal diet alone to serve as a "negative control." Others received in addition to the basal diet, quantitatively graded portions of the untreated air-dry yeast, or of alcoholic extracts of yeast, or of the corresponding extracted residues as sources of the antineuritic vitamin. This experimental feeding was continued for eight weeks.

The basal diet was a modification of the diet of Sherman and Spohn (15) into which autoclaved bakers' yeast (which had been heated three hours at 125° C.) was introduced at a level of 15 per cent, replacing an equal amount of cornstarch in the older diet. The composition of the diet used was as follows:

	Per cent
Casein, extracted with 60% alcohol	18
Salt mixture, Osborne and Mendel	4
Cod liver oil	3
Butterfat, filtered	7
Cornstarch	53
Autoclaved yeast (three hours at 125° C.)	15

Results of Feeding Experiments. Animals on the basal diet alone grew somewhat during the first 7 to 14 days, after which they declined rapidly and died in from 25 to 40 days. The 80 per cent alcohol extract of yeast

TABLE I

SUMMARY OF RESULTS, SHOWING SOLUBILITY OF THE ANTINEURITIC VITAMIN B OF YEAST IN ALCOHOL 80% BY WEIGHT

Yeast equivalents of supplements fed	Response of Young Rats to eight weeks feeding of Supplement Specified											
	Dry Bakers' Yeast				Alcohol (80%) Extract				Extracted Residue (80% alcohol)			
grams	No. rats	Gain grams	No. surviving 8 wks.	Average survival of rats dying earlier	No. rats	Gain grams	No. surviving 8 wks.	Average survival of rats dying earlier	No. rats	Gain grams	No. surviving 8 wks	Average survival of rats dying earlier
				days				days				days
No supplement (negative controls)	15	− 7.4	0	31.8	—	—	—	—	—	—	—	—
0.200	7	+ 1.2	2	37.0	9	− 3.9	0	38.4	—	—	—	—
0.400	9	+ 2.7	4	48.4	11	+ 3.3	8	38.0	3	−6.0	0	30.3
0.800	9	+47.6	9	—	9	+24.5	9	—	6	−7.3	2	34.0
1.600	—	—	—	—	10	+63.3	10	—	8	+1.7	4	46.2

appeared to contain much the larger part of the antineuritic vitamin originally present in the yeast. The extracted residue, fed in corresponding amounts, was relatively inactive. These results are shown in Table I and Figure 1.

The flattening of the growth curve during the experimental period in the case of the animals receiving the extract which had been concentrated

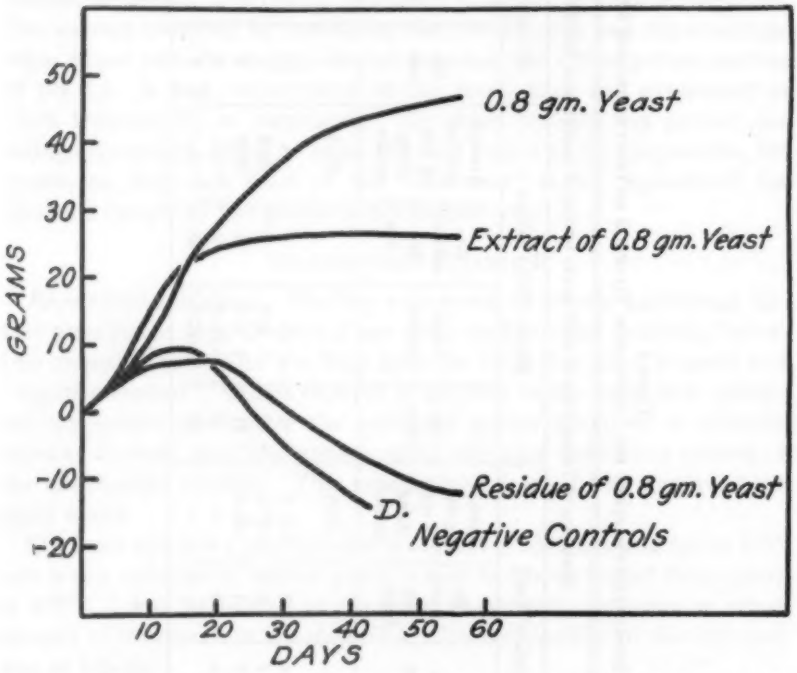


FIG. 1. Illustrating extraction of vitamin B (B_1) from yeast by alcohol of 80 per cent by eight.

at the temperature of the water-bath may have been due in part to a relative shortage of the thermolabile factor suggested by Reader (16) since the experiments here described were performed. Should further investigation confirm this suggestion, the vitamin B (B_1) of yeast would then appear somewhat more soluble in alcohol, as is also suggested by the fact that the residue here showed extremely little of this vitamin.

Solubility of Vitamin G

Parallel with these experiments on vitamin B, and on the same preparations, we studied also the solubility of vitamin G, extending the work to include the solubilities of vitamin G of yeast in alcohol of 95 and 60 per cent (by weight).

Experimental Methods. Healthy young albino rats were placed when 28 to 30 days old upon a basal diet adequate in other respects but freed as completely as was here practicable from the heat-stable factor to be studied. For this we used the Sherman and Spohn (15) diet modified by the addition of an alcoholic extract of ground whole wheat. This was

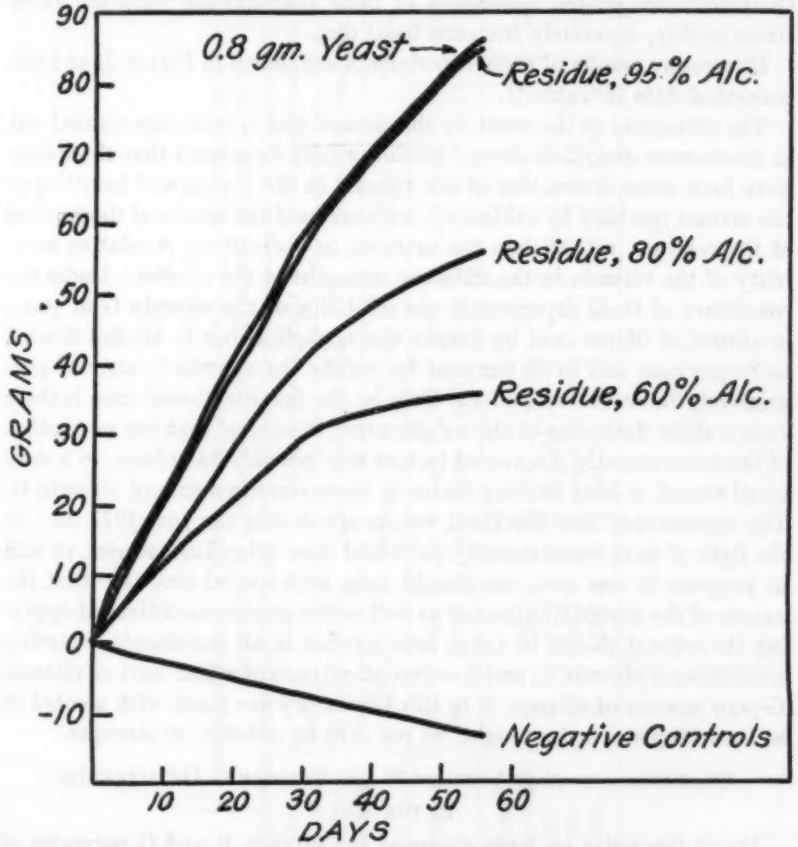


Fig. 2. Illustrating the relative lack of solubility of vitamin G in strong alcohol and its increasing solubility in alcohol diluted to 60 per cent by weight.

made by grinding wheat in the laboratory and extracting with *strong* alcohol, 80 per cent by weight. Experiments showed this extract to furnish an adequate supply of the antineuritic vitamin and apparently no measurable amount of vitamin G. In this way the heat-stable substance (although possibly supplied in small amounts) became the sole growth-limiting factor, so far as present knowledge indicates.

The rats were kept upon the basal diet alone until growth had ceased and a slight loss in weight had occurred. Following this depletion period, they received the basal diet with or without additions of the original dry yeast and of the various extracts and residues prepared as indicated above. Quantitatively graded allowances of these preparations were fed three times weekly, separately from the basal diet.

Illustrative results of these experiments are shown in Figure 2, and the numerical data in Table II.

The extraction of the yeast by the alcohol was in each case carried out in the manner described above. Finding reason to suspect that there may have been some destruction of the vitamin in the drying and handling of the extract (perhaps by oxidation), we have used the results of the feeding of the residues, rather than the extracts, as indications of relative solubility of the vitamin in the different strengths of the alcohol. Under the conditions of these experiments the solubility of the vitamin G of yeast in alcohol of 95 per cent by weight was negligible, but in alcohol diluted to 80 per cent and to 60 per cent by weight the vitamin G showed progressively increased solubility. Only in the last-mentioned case is there even a slight flattening of the weight curve to suggest that one or another of the more recently discovered factors may possibly have been, to a very small extent, a joint limiting factor in these measurements of vitamin G. The experiments here described were made during the year 1927–28. In the light of work more recently published from other laboratories, or still in progress in our own, we should note with special care: 1.—that the nature of the material extracted as well as the precise conditions of applying the solvent should be taken into account in all statements regarding solubilities of vitamin G, and 2.—that all extracts of wheat used as vitamin G-poor sources of vitamin B in this laboratory are made with alcohol of at least 80 per cent by weight, 84 per cent by volume, or stronger.

Observations upon Vitamin B and Vitamin G Deficiencies in the Rat

Up to this point we have discussed the vitamin B and G potencies of the materials under investigation on the basis of the amounts needed to

TABLE II

SUMMARY OF RESULTS OF FEEDING EXPERIMENTS SHOWING LACK OF SOLUBILITY OF VITAMIN G OF YEAST IN STRONG ALCOHOL AND ITS INCREASING SOLUBILITY IN ALCOHOL DILUTED TO 60% BY WEIGHT

Yeast equivalents of supplements fed	Average Gains in Eight Weeks on Supplement and Dose Specified													
	Dry Bakers' Yeast		95% Alcohol Extraction				80% Alcohol Extraction				60% Alcohol Extraction			
	Untreated		Residue		Extract		Residue		Residue		Residue		Extract	
grams	No. rats	Gain grams	No. rats	Gain grams	No. rats	Gain grams	No. rats	Gain grams	No. rats	Gain grams	No. rats	Gain grams	No. rats	Gain grams
No Supplement (negative controls)	24	−10.9	—	—	—	—	—	—	—	—	—	—	—	—
0.200	12	+15.6	10	+17.0	—	—	6	+11.0	—	—	—	—	—	—
0.400	16	+44.5	11	+46.8	3	− 5.3	7	+28.0	—	—	7	+14.1	6	+12.6
0.800	14	+84.2	10	+84.2	6	− 7.5	6	+55.8	6	− 6.5	6	+33.3	6	+26.2

induce similar growth responses under carefully controlled conditions. In common with several other investigators we have found that feeding graded amounts of either vitamin as supplements to a suitable basal diet, results in a series of weight curves graded from those of the "negative control" to those approaching normal growth.

The judgments which we have based on these gain curves have been confirmed by the observation of certain pathological symptoms corresponding to varying degrees of the vitamin deficiencies.

An allowance of vitamin B too small to maintain weight, but sufficient to prolong life, was attended quite regularly with appearance of the classic symptoms of polyneuritis; an allowance of vitamin B sufficient to maintain weight was characterized by a subacute type of polyneuritis; a still larger allowance promoted growth without the appearance of polyneuritis; but a practically complete deprivation of vitamin B (B_1) resulted in death before apparent symptoms of polyneuritis developed. The symptoms of vitamin B deficiency as here observed in the rat have been discussed in another paper (17).

Within certain limits, the restriction of vitamin G (B_2) resulted in pellagra-like symptoms such as have been described by Goldberger and his coworkers, and by Chick and Roscoe. In cases of almost complete deprivation of vitamin G (B_2) death usually occurred without the development of conspicuous skin symptoms. In general our experiments indicated that in the condition resulting from almost complete deprivation of this vitamin, the eyes, nose, mouth, alimentary tract, and perhaps kidneys are apt to show more pronounced abnormalities than the skin; whereas with a less complete deprivation and a somewhat longer course of the deficiency disease, the abnormalities of the skin are apt to become much more prominent. Sometimes, on diets extremely deficient in vitamin G, symptoms may appear around the eyes, mouth or nose as early as the fifth week; but frequently only after a longer time. Often, where there is a measurable but restricted amount of vitamin G supplied, there is no development of superficially visible lesions within the eight weeks experimental period which we have been accustomed to use in quantitative comparisons of vitamin values; but sooner or later the condition, described as pellagra-like by Goldberger *et al* and by Chick and Roscoe (19), develops regularly enough to leave no doubt that this should be regarded as a characteristic nutritional-deficiency condition—a G-avitaminosis—whether or not an infection is also involved; and whether or not it prove to be a real analogue of pellagra.

The first of the accompanying photographs (Fig. 3) permits comparison of the condition of two typical experimental animals, one of which had received, as source of vitamin G, the residue and the other the extract

FIG. 3. Illustrating abundance of vitamin G in strong-alcohol-extracted yeast, paucity of this vitamin in the strong-alcohol extract and eye symptoms of vitamin G deficiency in the young rat.

FIG. 4. Showing typical lesions around the eyes, nose, and mouth as they develop in young rats when almost completely deprived of vitamin G.

from a treatment of yeast with strong alcohol. In all other respects their nutritional and other environmental history was the same. The residue,

rich in vitamin G, resulted in normal growth and development; the effect of the almost complete absence of vitamin G from the corresponding strong alcohol extract is shown in the retarded growth and the characteristic condition around the eye of the smaller rat (Laboratory No. L316). Sometimes the loss of hair around the eyes is accompanied or followed by the development of a surrounding rim of scabby discoloration giving rise to the term "spectacle-like" condition. Soreness of the nose and mouth often develops with, or soon after, that around the eyelids, as shown in Fig. 4. These are the most characteristic appearances in the cases of almost complete deprivation of vitamin G in young animals.

Professor Alwin M. Pappenheimer of the College of Physicians and Surgeons, Columbia University, was kind enough to examine one animal, L316, which presented a picture of severe deficiency. Dr. Pappenheimer's statement of the condition follows:

> Sections through the eye show a very slight keratitis, with corneal corpuscles, a few polymorphonuclears and some new formed blood channels. The lacrymal glands in one section show an acute inflammatory process; the lumen of the glands containing numerous polymorphonuclear leucocytes. This may be the cause of the loss of hair above the eyelids; possibly there is an alteration of the lacrymal secretion.
>
> A section through the oedematous eyelid shows a desiccation of the epidermis, oedema of the cellular tissue and a slight inflammatory reaction. The lip is the seat of a chronic ulcer. The epithelium is missing over a considerable area; the base of the ulcer is formed by chronically inflamed granualation tissue.
>
> The stomach shows a papillary epithelial hyperplasia, such as Dr. Larimore and I described in rats on variously deficient diets, and which we attributed to hair ingestion.

The pronounced "pellagra-like" dermatitis with red inflamed paws, or "saddle-like" areas of baldness and dermatitis on the shoulders or back, we have found to be more characteristic of cases in which the deprivation of vitamin G was less early or less complete with a longer survival period and therefore permitted more time for the gradual development of the dermatitis. A moderate development of this type of case is shown in Fig. 5, a photograph taken at the age of 150 days of rat No. L174 which had received a restricted allowance of vitamin G from the age of 4 weeks to that of 18 weeks after which this allowance was discontinued for 6 weeks. By the end of this latter period, or about two weeks after the time at which the photograph was taken, the animal was almost denuded of fur, the skin was dark red, wrinkled and old-looking; the back was covered by a dirty yellow-brown crust which presented a somewhat cheesy appearance; one eye was sore; the front paws were inflamed. This animal was subsequently cured by the feeding of a yeast preparation as source of vitamin G.

In a case of less acute but longer continued shortage of vitamin G, there was gradual but extensive development of a mild dermatitis and loss of hair with increasing weakness terminating in death at eight months of age. The final appearance of this animal is shown in Figure 6.

FIG. 5. Loss of hair accompanying mild or latent but extensive dermatitis more characteristic of later or less complete deprivation of vitamin G.

FIG. 6. Showing condition resulting from a long continued subnormal intake of vitamin G; born 1/18/1927, died 9/12/1927. (From an experiment conducted in this laboratory by Dr. J. H. Axtmayer and Dr. E. L. Chase).

Like Chick and Roscoe (19) we have found that the rough, red, swollen paws, which probably constitute the chief or most obvious reason for the designation of the G-avitaminosis as a "pellagra-like" condition, are by no means characteristic of those more acute cases of this nutritional

deficiency which develop upon diets well freed from vitamin G; but are seen, rather, in cases of less complete but long-continued vitamin G deficiency.

Curative Experiments. When animals which have developed typical lesions of G-avitaminosis are given food containing adequate amounts of vitamin G, the characteristic result is a rapid and striking change during the first few days, after which the healing continues, less rapidly but steadily, until apparently complete health is restored. Several weeks are sometimes required for complete cure. Sometimes too there may be a short period at the beginning of the cure in which the skin appears worse than before, because of the sloughing off of skin which (under the shortage of vitamin G) had gradually degenerated. Two of the curative experiments may be briefly described as follows:

Rat L294, after 15 weeks of shortage (not complete deprivation) of vitamin G was described as showing: rims of inflammation around the eyes; hair thin; small, dry, cream-colored scales noticeable over the skin of the back. Five weeks later these scales had been replaced by yellowish crusts which extended over a large part of the body; the fur was short, matted and dirty-looking; the legs almost denuded, and showing redness and roughness of the skin; the animal was weak and seemed indifferent to its surroundings, remaining in a humped posture with head low and the feet close together. The feeding of autoclaved yeast was then begun in allowances equivalent to one gram per day. Within 48 hours the animal was more active but showed distress as the skin was peeling off in large flakes; by the third day after the restoration of vitamin G to his food supply the animal was distinctly improved, alert and interested in its surroundings, though the peeling continued until the seventh day; after 10 days the skin seemed normal and new fur was visible; after 4 weeks the animal had doubled its weight, and appeared entirely normal, its skin smooth and pink, its fur soft, thick and silky.

Rat L307, after 15 weeks on a diet very poor in vitamin G, had not changed in weight (44 grams) and was described as showing: raw and inflamed region around nose and mouth; eyes surrounded by bald, swollen rims; diarrhea; bloody urine at times; extreme weakness. Within 24 hours after giving food rich in vitamin G the condition around the eyes was greatly improved; within the next three days extensive desquamation left the animal's face quite bald and smooth; normal alertness and activity returned; new fur now began to appear on the face while extensive peeling progressed over much of the body. After 10 weeks the cure seemed complete, the animal sleek and well, eyes normal, skin smooth and pink, fur long, thick and glossy; weight 176 grams, (quadrupled during the 10 weeks of vitamin G feeding).

Appetite. In comparing the results of diets substantially alike and adequate in other respects but almost completely freed from vitamin B or vitamin G, respectively, it was found that the loss of appetite and resulting decrease of food consumption which have frequently been noted in connection with studies of the undifferentiated vitamin B complex, are very much more pronounced in the case of deprivation of the present vitamin B (B_1) than of vitamin G (B_2), as may be seen from Fig. 7, which shows the average results of 12 animals on each diet. In fact the difference

here is so pronounced that it seems logical to regard the characteristic or truly specific effect upon appetite as a property of vitamin B (B_1) and to regard the diminishing food consumption during the G-avitaminosis as probably only the usual concomitant of the weakness, lack of tone, and increasing torpidity of the experimental animals. The sharp decline in the food intake in the former case supports the older findings that there

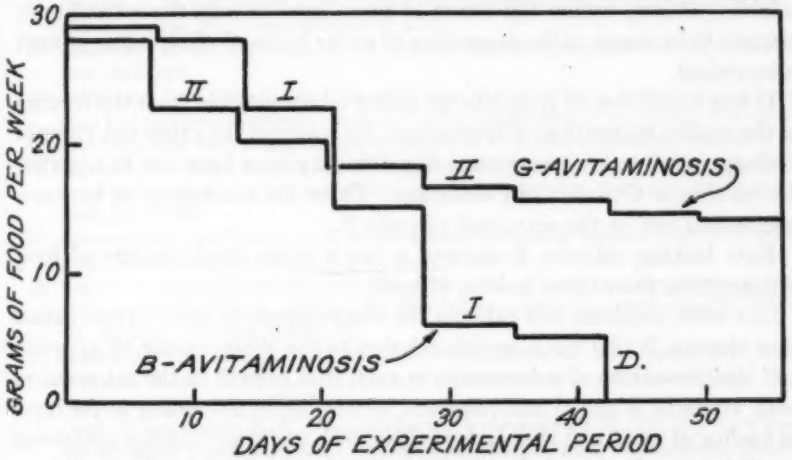


FIG. 7. Showing average food consumption of comparable groups of 12 experimental animals each, one group deprived of vitamin B and the other of vitamin G. In all cases the animals were placed upon the experimental diet at the age of 28 to 30 days.

is a more or less specific failure of appetite in animals receiving diets deficient in the antineuritic vitamin, which results in a more rapid decrease of food intake than that attributable to mere general weakness and apathy.

Another striking illustration is found in the case of animals receiving the extracted yeast residue as a supplement to the basal diet. When fed as the sole source of the antineuritic vitamin, it was uniformly and persistently refused unless the food cup was removed from the cage until the dose was eaten; whereas when portions of an identical sample were fed to rats as a source of vitamin G as supplement to a basal diet containing an ample amount of antineuritic vitamin, the extracted yeast residue was greedily eaten. This would seem to indicate that palatability was not the determining factor in the refusal of young rats to eat the food supplied; but, as others have also found, that vitamin B is definitely related to appetite as a physiological function.

Summary and Conclusions

Dried bakers' yeast yielded a much larger proportion of its vitamin B (B_1) than of its vitamin G (B_2) to extraction with 80 per cent alcohol.

In 95 per cent alcohol, as used in these experiments, no measurable amount of the vitamin G of yeast was dissolved; in 80 per cent alcohol the solubility was appreciable; in 60 per cent alcohol the vitamin G was more soluble. Hence, within the limits of these experiments, the solubility of vitamin G increases as the proportion of water in the alcohol: water solvent is increased.

It was found best to judge the solubility of the vitamin from the feeding of the residue rather than of the extract, for a part of the extracted vitamin G disappeared and it is suggested that this may have been due to a partial destruction of vitamin G by oxidation. There did not appear to be a corresponding loss of the extracted vitamin B.

Rats lacking vitamin B showed a much more rapid decline of food consumption than those lacking vitamin G.

Our work confirms and extends the observations of other investigators that vitamin B (B_1) has a specific relation to the maintenance of appetite and the prevention of polyneuritis in rats; that growth in the rat requires both vitamins B and G and responds, within limits, according to the level of feeding of each; and that lack of vitamin G, under sufficiently controlled conditions, quite regularly produces in the rat the deficiency disease designated as pellagra-like by Goldberger and his associates and by Chick and Roscoe, though the particular type of skin lesion which this term suggests is not characteristic of the cases of most complete deprivation of vitamin G.

Whether the observed variability of symptoms of the G-avitaminosis is connected with multiple nature of vitamin G is a question still under investigation.

References

1. Mitchell, H. H., On the identity of the water-soluble growth-promoting and the antineuritic vitamine. *Jour. Biol. Chem.*, 1919, XL, 399.
2. Sherman, H. C., and Smith, S. L., The Vitamins. New York., 1922.
3. Kruse, H. D., and McCollum, E. V., Biochemical investigation of vitamin B. *Physiol. Reviews*, 1929, IX, 126.
4. Fraser, H., and Stanton, A. T., The etiology of beriberi. *Studies Inst. Med. Research, Fed. Malay States*, No. 12, 1911.
5. Cooper, E. A., On the protective and curative properties of certain foodstuffs against polyneuritis induced in birds by a diet of polished rice. *Jour. Hygiene*, 1914, XIV, 12.
6. Funk, C., The preparation from yeast and certain foodstuffs of the substance the deficiency of which in the diet occasions polyneuritis in birds. *Jour. Physiol.*, 1912, XLV, 75.

7. McCollum, E. V., and Kennedy, C., The dietary factors operating in the production of polyneuritis. *Jour. Biol. Chem.*, 1916, XXIV, 491.
8. McCollum, E. V., and Simmonds, N., A study of the dietary essential water-soluble B, in relation to its solubility and stability towards reagents. *Jour. Biol. Chem.*, 1918, XXXIII, 55.
9. Osborne, T. B., and Wakeman, A. J., Extraction and concentration of the water-soluble vitamine from brewer's yeast. *Jour. Biol. Chem.*, 1919, XL, 383.
10. Drummond, J. C., A study of the water-soluble accessory growth-promoting susbtance in yeast. I. *Biochem. Jour.*, 1917, XI, 255.
11. Osborne, T. B., and Mendel, L. B., A critique of experiments with diets free from fat-soluble vitamine. *Jour. Biol. Chem.*, 1920, XLV, 277.
12. Kinnersley, H. W., and Peters, R. A., Antineuritic yeast concentrates. I. *Biochem. Jour.*, 1925, XIX, 820.
13. Goldberger, J., Wheeler, G. A., Lillie, R. D., and Rogers, L. M., A further study of butter, fresh beef and yeast as pellagra preventives, with consideration of the relation of factor P-P of pellagra (and black tongue of dogs) to vitamin B. *U. S. Pub. Health Rep.*, 1926, XLI, 297.
14. Sherman, H. C., and Sandels, M. R., Experiments with reference to the more heat-stable factor in the vitamin B group (factor P-P, vitamin B_2 or G). *Proc. Soc. Exper. Biol. and Med.*, 1929, XXVI, 536.
15. Sherman, H. C., and Spohn, A., A critical investigation and an application of the rat growth method for the study of vitamin B. *Jour. Amer. Chem. Soc.*, 1923, XLV, 2719.
16. Reader, V., A second thermolabile water-soluble accessory factor necessary for the nutrition of the rat. *Biochem. Jour.*, 1929, XXIII, 689; Further evidence of a third accessory "B" factor. *Biochem. Jour.* 1930, XXIV, 77.
17. Sandels, M. R., Experimental nutritional polyneuritis in the rat. *This Journal*, 1930, II, 409.
18. Smith, M. T., and Hendrick, E. G., Some nutrition experiments with brewers' yeast. *U. S. Public Health Rep.*, 1926, XLI, 201.
19. Chick, H., and Roscoe, M. H., The dual nature of water-soluble B. II. The effect upon young rats of vitamin B_2 deficiency and a method for the biological assay of vitamin B_2. *Biochem. Jour.*, 1928, XXII, 790.

THE INFLUENCE OF BULK IN THE DIET UPON FECAL CALCIUM AND PHOSPHORUS*

By

LEAH ASCHAM

(From the Laboratory of Physiological Chemistry, Yale University, New Haven, and the Laboratory of the Division of Home Economics, Kansas State Agricultural College, Manhattan.)

Received for Publication—May 23, 1930

INTRODUCTION

THE effect that roughage in the diet may have upon the utilization of the various nutrients has not been extensively studied, though crude fiber or some other feces-former has constituted a part of many experimental diets. Recently there appeared an article by Whitacre, Willard and Blunt (1) upon the influence of fiber on the nitrogen balance and the fat in the feces. They concluded that increased bulk tends to reduce the utilization of nitrogen but does not have the same effect upon the utilization of fat.

Calcium metabolism and the factors influencing it have been subjects of investigation for many years and yet in all these numerous studies little attention has been given to possible losses of calcium through increase in the fecal mass. In surveying the literature upon the subject of bulk as a factor in calcium metabolism, one finds only scattering statements and these are reports of incidental observations. Neither has there been much attempt to formulate any definite hypothesis concerning the physiological action of bulk in its relation to calcium excretion. As early as 1880 Voit (2) stated that there was loss of calcium through excessive production of feces. Hart and Steenbock (3) found that with bran the bulk of the fecal excretion was large, and that this might decrease the amount of calcium absorbed from the ration; whereas McLaughlin (4), in her study on calcium utilization, fed unusually large quantities of spinach, and no hindrance to calcium assimilation through the presence of fiber or oxalate was demonstrated. Hart, Steenbock, Kletzien and Scott (5) reported that a lag in intestinal excretion is accompanied by large negative calcium balances.

Givens and Mendel (6) added agar to a food mixture so that a daily

* The data reported in this paper are taken from the dissertation of Leah Ascham presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Yale University June, 1929.

evacuation of the bowel might lessen the possibility of reabsorption of calcium. In an investigation upon the utilization of the calcium in carrots and spinach, McCluggage and Mendel (7) discovered that the fecal volume increased 90 to 100 per cent when the vegetables were fed. There was also increased calcium loss though it was not proportional to the fecal output. The data of Findlay, Paton and Sharpe (8) show that calcium formed a fairly fixed percentage of the dry weight of the feces and since the weight of the feces varied considerably from day to day, the calcium ouput varied also. It was concluded by Telfer (9) that calcium and fat directly controlled the size of the fecal mass because a decrease in either of these factors resulted in a diminution in the excretion of calcium soap. This effect upon the volume of the feces was not due to the soaps *per se* but to the fact that they incorporated other intestinal contents. Thus calcium was a controlling factor in the formation of feces and not bulk a controlling factor in calcium excretion.

Sjollema conducted two experiments (10, 11), one with cod liver oil, the other with crude fiber, in which he observed an increased fecal calcium with an increased fecal volume. He suggested that calcium might be concerned with the function of the intestines especially in connection with feces formation. Should volume of feces have the deleterious effect of increasing the calcium output, it might be difficult to maintain positive balances in both man and animal, especially since foods which introduce much crude fiber into the diet are also low in calcium content, thus making the probability of negative balances even greater. However, when one studies Sjollema's data there is evidence of so many uncontrolled variables that his conclusions seem unsound. The diets were poorly constructed, the same conditions were not maintained throughout an entire experiment and, further, the conclusions were based upon results obtained on one animal.

Since it was impossible to find anything conclusive concerning the relation of volume of feces to calcium excretion, it seemed worth while to investigate the subject and determine, if possible, whether bulk could be a hindrance to the maintenance of positive calcium balances. Phosphorus determinations were made to note the possible effect exerted by bulk upon this element also. The feeding technique of Cowgill (12) was followed.

Experimental Procedure

Four adult, female fox terrier dogs were used throughout the entire series of these experiments and one adult, female mongrel for a portion of the time. The dogs gradually increased in weight during the whole period and

always appeared to be in good physical condition. Their appetites varied considerably from time to time but apparently this was due in every case to some factor other than mere physical state. The dogs were housed in a room where ventilation and temperature were well regulated but which was inaccessible to sunlight. They were kept in metabolism cages permitting the separate collection of feces and urine.

Method Used for Determining the Food Intake. In order to ascertain the food intake which would just maintain the dogs while they were in the metabolism cages, the surface area of each was determined according to the formula devised by Cowgill and Drabkin (13) while the calories required and the calories fed were computed from figures given by Cowgill. He stated that "dogs given all they desire of our artificial casein diets adjust their food intake so as to maintain a fairly constant energy intake of approximately 64 calories per hour per square meter of surface. Dogs may be maintained satisfactorily in the laboratory when fed at an energy level approximately 15 to 20 per cent below this figure, and when so fed a complete diet, eat every bit of the food offered."

Definition and Composition of the "Food Unit." The term "food unit" as used throughout this study signifies a food mixture consisting of constant fixed amounts of casein, sugar, lard, and salt mixture but of varying proportions of cellulose-flour and agar, used as sources of roughage. When the amount of roughage changed, the weight of the "food unit" changed also, but the nutritive value of the "unit" was not affected and hence the total food intake of each dog remained the same even though the total grams of ingested material varied.

The composition of the food unit by periods is given in Table I.

TABLE I
COMPOSITION OF THE "FOOD UNIT"

	Casein washed	Casein unwashed	Sugar	Lard	Salt mixture	Cellulose-flour	Agar
	gm	gm.	gm.	gm.	gm.	gm.	gm.
Period I	20	10	32	18	2	18	
II	20	10	32	18	2	18	
III	7	23	32	18	2	3	
IV	7	23	32	18	2	3	
V	7	23	32	18	2	18	
VI	10	20	32	18	2		2
VII	28	2	32	18	2		12

Distilled water was used at all times.

The ration was prepared separately for each dog during each experimental period. Essentially the same food mixture, except for the percentage of roughage, was fed during the entire time the dogs were in the laboratory, a period of nearly six months.

As commercial casein contained more calcium than was desired in the diet, the crude casein was treated with 0.0001 N HCl twice a day for a week, then washed with distilled water and 95 per cent alcohol. Three lots of washed casein contained 0.041, 0.039, and 0.027 per cents of calcium, respectively. Cellulose flour[1] used for roughage yielded no ash. It contained 37.5 per cent crude fiber. The agar contained 0.665 per cent of calcium and 0.035 per cent of phosphorus. One cc. of cod liver oil as the sole source of both the antixeropthalmic and antirachitic vitamins was given daily to each dog by dropping it upon the food mixture in the food pan. From 0.8 to 1.0 gram of wheat germ extract[2] per kilogram per day was given as a source of vitamin B. To insure its complete consumption it was dissolved in a small amount of water. The salt mixture consisted of 234.0 gms. NaCl, 544.0 gms. KH_2PO_4, 70.6 gms. Mg-citrate, 23.9 gms. Fe-citrate, and 4.0 gms. KI.

Calcium and phosphorus determinations were made on six-day samples of urine and feces; nitrogen determinations upon three-day samples of urine. The analytical methods employed were that of McCrudden (14) for calcium, the volumetric method of the Association of Official Agricultural Chemists for phosphorus, and of Kjeldahl for nitrogen.

Results

The diet was so planned that the only change ever made in the composition of the "food unit" was in the kind and amount of roughage with the possible exception of minor variations in the calcium-phosphorus ratio and it will be noted in Table II that the total food consumption for each dog during the cellulose flour experiments was essentially constant. The different amounts of the bulk factor were always introduced gradually but even then the dogs reacted very noticeably to alterations in the food mixture; their objection to a new ration seemed to be due entirely to its physical texture. Even greater difficulties were encountered with the agar diet, the food intake of only one dog remaining normal. It appeared that both consistency and flavor were disagreeable to the dogs. Since it was

[1] Celluflour, Chicago Dietetic Supply House, Chicago.

[2] Vitavose E. R. Squibb & Sons, New York.

desired to use the same number of "food units" for each dog during each of the experimental periods, the intervals between them were much prolonged and hence the data collected represent a study over a protracted period of time.

It was assumed from the nitrogen balances of the first four periods that it would be unnecessary to continue the nitrogen analyses. Without doubt negative balances did occur during the agar periods but it seemed inadvisable to change the composition of the "food unit" by introducing more casein to compensate for the lessened nitrogen intake. Such complications might have been much greater than those arising from the decreased food intake.

Neither the calcium nor phosphorus intakes were kept exactly constant. The percentage of phosphorus in casein alone varied enough to make it impossible to keep the phosphorus content of the food as uniform as that of the calcium. Of the two elements, the constancy of the latter was the chief consideration in the organization of the diets. As a consequence the Ca/P ratio of the intake was not identical throughout the different periods. However, it would seem that the differences, small as they were, could not have affected the metabolism of these elements in the least. Dogs *might* be more sensitive than rats to fluctuations in the Ca/P ratio but it is thought that, so far as rats are concerned, probably variations of this ratio in their normal diets, ranging anywhere from 1.0 to 2.0, are of minor importance (15).

All of the feces were well formed, even those where the high percentage of cellulose flour was used in the ratio. The dogs varied somewhat in the frequency of defecation, but in general there were from one to two defecations per day with both the high cellulose flour and the high agar diets. There was a slight tendency for more frequent defecations with the low agar diet than with the low cellulose flour diet, though with either they were not more frequent than every second day and an occasional three-day interval occurred with cellulose flour.

Table II summarizes the data for calcium and phosphorus upon the common basis of the "food unit." It will be noted that there are wide fluctuations in the data not only for the different dogs upon the same diet but also for the same dog upon different diets. Averages tabulated in Table III give a somewhat different picture. With one exception (Dog 2), the excretions of both calcium and phosphorus are greater during the periods of high roughage.

TABLE II

CALCIUM AND PHOSPHORUS UTILIZATION PER "FOOD UNIT"

Period and Animal No.	Total "food units" per period	Feces		Calcium					Phosphorus				
		Moist weight	Dry weight	Per cent in feces	Intake	Feces	Urine	Balance	Per cent in feces	Intake	Feces	Urine	Balance
		gm	gm.		mg.	mg.	mg.	mg.		mg.	mg.	mg.	mg.
High cellulose flour													
Period I, Dog 1	9.6	32.7	20.8	0.69	97	144	7	− 54	0.50	486	104	521	−139
" 2	7.8	26.2	19.7	0.74	97	146	3	− 52	0.48	486	95	498	−107
" 3	7.2	34.1	20.5	0.62	97	127	13	− 43	0.52	489	107	536	−154
" 4	10.5	32.0	21.4	0.75	98	161	10	− 73	0.49	491	105	554	−168
" 5	7.2	27.4	19.5	0.67	97	131	12	− 46	0.51	489	99	551	−161
Period II, Dog 1	9.6	24.9	20.5	0.71	97	146	16	− 65	0.50	490	103	517	−130
" 2	7.8	20.3	16.9	0.69	98	117	5	− 24	0.46	486	78	540	−132
" 3	7.2	32.4	19.3	0.57	97	110	11	− 24	0.43	489	83	485	− 79
" 4	10.8	32.6	20.8	0.75	98	156	28	− 86	0.44	491	92	547	−149
" 5	7.2	31.4	21.5	0.65	97	140	—	—	0.49	489	105	533	−149
Period V, Dog 2	7.8	32.3	20.1	0.60	98	121	23	− 46	0.36	556	72	462	+ 22
" 5	7.1	27.9	19.8	0.61	98	120	13	− 35	0.43	559	85	505	− 31
Low cellulose flour													
Period III, Dog 1	9.9	9.4	5.1	2.36	98	120	6	− 28	1.44	528	73	499	− 44
" 2	7.4	5.5	4.3	2.70	98	116	11	− 29	1.77	523	76	495	− 48
" 3	7.4	9.1	5.2	2.14	98	111	13	− 26	1.55	523	81	469	− 27
" 4	10.9	9.9	4.2	2.63	98	110	17	− 29	1.74	525	73	472	− 20
" 5	7.4	9.0	4.8	2.53	98	121	9	− 32	1.14	523	55	453	+ 15

Period IV, Dog	1	9.9	8.8	6.1	2.47	99	150	17	− 68	1.67	562	102	567	−107
"	2	8.6	8.6	5.4	2.67	98	144	19	− 65	1.85	554	100	380	+ 74
"	3	7.4	6.7	3.8	2.44	98	93	5	0	1.65	558	63	459	+ 36
"	4	10.9	10.5	3.7	3.04	98	112	5	− 19	1.98	560	73	477	+ 10
"	5	7.4	9.1	4.8	2.52	98	121	11	− 34	1.86	558	89	466	+ 3
High agar														
Period VII, Dog	1	6.4	37.0	10.9	1.36	103	148	12	− 57	0.86	592	94	464	+ 34
"	2	2.6	*	16.7	1.30	113	217	26	−130	0.72	684	120	802	−239
"	3	4.0	39.8	14.0	1.22	106	171	15	− 80	0.84	623	118	562	− 57
"	5	7.0	43.3	13.0	1.05	102	137	10	− 45	0.75	588	98	508	− 18
Low agar														
Period VI, Dog	1	8.9	10.5	3.9	3.32	101	129	13	− 51	2.25	593	88	520	− 15
"	2	5.0	*	5.0	3.28	105	164	23	− 82	2.01	603	101	593	− 91
"	3	6.4	5.2	2.8	3.36	102	94	18	− 12	2.16	607	60	535	+ 12
"	5	7.5	11.8	4.1	2.84	101	116	12	− 27	2.07	600	85	532	− 17

* Found in water pans.

TABLE III

AVERAGE FECAL CALCIUM AND PHOSPHORUS FOR EACH EXPERIMENTAL DIET

Diet	Calcium						Phosphorus					
	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	General Average	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Average
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
High cellulose flour	144	127	119	159	130	135.8	103	81	95	98	96	94.6
Low cellulose flour	135	131	103	112	122	120.6	88	88	72	73	72	78.6
High agar	148	217	170	—	135	167.5	94	120	117	—	95	106.5
Low agar	129	163	95	—	115	127.0	87	100	61	—	84	83.0

Discussion and Conclusions

The dogs were kept in a state of negative calcium balance at all times as it was thought that if there were differences in calcium excretion in the various periods, these differences would thus be more evident. If the organism does excrete calcium from the bones more easily than it produces feces with low calcium content, as stated by Sjollema (10), it was believed that this would be demonstrated more clearly by the maintenance of negative balances. It was surmised that even normal fluctuations of a positive balance might possibly be great enough to make interpretations of the physiological function suggested by him very difficult.

Calcium-Phosphorus Ratio. In the light of recent studies, the Ca/P ratio of the diets used in this investigation was probably far from adequate even for adult dogs receiving cod liver oil daily. Whether it vitiated the results in this type of experiment is hard to determine. In another experiment conducted later in the year upon these same dogs, the Ca/P ratio was increased somewhat, and the balances of both elements were for the most part positive, but still there were the same wide fluctuations in their elimination. (In the experiment cited the amount of bulk was constant throughout.) It is difficult to determine to what extent the decreased food intake during the agar experiments affected the results. It has been demonstrated that starvation will cause healing in rickets (16) although rats upon restricted intakes of rickets-producing food show the usual signs of the disease (17).

Calcium-Phosphorus Metabolism. In view of the wide variations found in the amount of calcium in the feces not only of the different dogs upon the same diets, but also of each individual dog upon similar diets, it becomes somewhat difficult to compare the results found in the different series and consequently to draw any conclusions as to the effect of bulk upon the output of calcium in the feces. Meigs and coworkers (18) have called attention to the capricious variations in the quantities of calcium and phosphorus found in the feces of cows in experiments where there are no recorded changes in the diets or in other conditions to explain the variations. They state that these irregularities may be due to real differences in the rate of absorption of these elements or to the irregularities in the rate at which fecal matter is discharged, as well as to the disturbances in digestive processes arising from changes in routine. As the digestive tract of the dog differs from that of the cow, the same reasons for the differences in the rate of absorption and of discharge may not apply. It was interest-

ing to observe, however, that the rates of discharge of the feces were not uniform with any of the dogs.

When averages and not variations are considered there does seem to be a tendency for the calcium, as well as the phosphorus in the feces, to be increased during the periods of increased bulk. The effect of the agar in this respect was apparently greater than that of cellulose flour which might be attributed to several factors. The action of the flour in the intestinal tract probably is quite different from that of agar, since the former functions as a feces-former because of its indigestible residues, the latter because of its water-absorbing power, and as a consequence the percentage of moisture in the agar feces greatly increased their volume (Table II), an important factor in intestinal activity.

In the agar series, unfortunately, it seemed necessary to introduce the smaller quantity into the ration first. Hence the question arises as to whether the animals had been in negative balance for so long that their stores of calcium and phosphorus were being gradually depleted, a depletion merely augmented by the increased amount of agar, or whether the losses of these elements would have been of the same magnitude had the agar series been reversed. As agar furnished part of the calcium in these diets (phosphorus was negligible), there arises also the question of availability.

There is evidence from the data presented in this paper, though it is not altogether conclusive, that bulk may increase the fecal calcium. There is also evidence that bulk influences the excretion of phosphorus in the feces. Consequently one is led to conclude that it is not possible to assign a special function to calcium. If it has a physiological action of importance in the intestines, this investigation has not shown it.

The writer expresses her indebtedness to Professor Lafayette B. Mendel at whose suggestion and under whose guidance the investigation was carried on. Grateful acknowledgements are extended to Professors Arthur H. Smith and George R. Cowgill for many valuable suggestions; to Professor W. L. Latshaw in charge of the analytical laboratories of the Kansas Agricultural Experiment Station and to Dr. Margaret M. Justin, Dean of the Division of Home Economics of the Kansas State Agricultural College, for their cooperation during the progress of the experimental work.

BIBLIOGRAPHY

1. Whitacre, J., Willard, W., and Blunt, K., *This Journal*, 1929, II, 187.
2. Voit, E., *Zeit. Biol.*, 1880, XVI, 117.
3. Hart, E. B., and Steenbock, H., *Jour. Biol. Chem.*, 1913, XIV, 75.

4. McLaughlin, L., *Jour. Biol. Chem.*, 1927, LXXIV, 455.
5. Hart, E. B., Steenbock, H., Kletzien, S. W., and Scott, H., *Jour. Biol. Chem.*, 1927, LXXI, 271.
6. Givens, M. H., and Mendel, L. B., *Jour. Biol. Chem.*, 1917, XXXI, 421.
7. McCluggage, H. B., and Mendel, L. B., *Jour. Biol. Chem.*, 1918, XXXV, 353.
8. Findlay, L., Paton, D. N., and Sharpe, J. E., *Quart. Jour. Med.*, 1920–21, XIV, 354.
9. Telfer, S. V., *Quart. Jour. Med.*, 1924, XVII, 245.
10. Sjollema, B., *Jour. Biol. Chem.*, 1923, LVII, 255.
11. Sjollema, B., *Jour. Biol. Chem.*, 1923, LXVII, 271.
12. Cowgill, G. R., *Jour. Biol. Chem.*, 1923, LVI, 725.
13. Cowgill, G. R., and Drabkin, D. L., *Amer. Jour. Physiol.*, 1927, LXXXI, 36.
14. McCrudden, F., *Jour. Biol. Chem.*, 1911–12, X, 187.
15. Shohl, A. T., Bennett, H. B., and Wed, K. L., *Jour. Biol. Chem.*, 1928, LXXIX, 257.
16. McCollum, E. V., Simmonds, N., Shipley, P. Q., and Park, E. A., *Bull. Johns Hopkins Hosp.*, 1922, XXXIII, 31.
17. Shohl, A. T., and Bennett, H. B., *Jour. Biol. Chem.*, 1927, LXXIV. 247.
18. Meigs, E. B., Turner, W. A., Harding, T. S., Hartman, A. M., and Grant, F. M., *Jour. Agri. Research*, 1926, XXXIII, 833.

THE AUTO-OXIDATION OF FATS WITH REFERENCE TO THEIR DESTRUCTIVE EFFECT ON VITAMIN E

By

MARIAN J. CUMMINGS* AND H. A. MATTILL

(From the Department of Vital Economics, University of Rochester, Rochester, N.Y., and The Biochemical Laboratory, State University of Iowa, Iowa City.)

Received for Publication—May 31, 1930

THE oxidation of unsaturated fats by atmospheric oxygen causes the formation of substances which impart to those fats a characteristic acrid odor, usually described as rancid. Rancidity is difficult to define; some of the products formed have been identified, among them free fatty acids, aldehydes, ketones and peroxides; various theories have been advanced to explain the mechanism of their formation, but there is no agreement upon a satisfactory criterion for rancidity.

Fats differ greatly in the tendency to become rancid, and the studies of Holm and his coworkers (1, 2) on the suceptibility of fats to oxidation have indicated some of the governing influences. Moisture prolongs the induction period and thus retards the auto-oxidative process; certain compounds containing the hydroxyl group have a powerful retarding effect; unsaturated fatty acids produced or liberated during the process are catalytic; the presence of peroxides or "moloxides" allows oxidation to proceed even *in vacuo*; the induction period is markedly shortened by exposure of the fat or oil to ultraviolet light.

The auto-oxidation of unsaturated oils or fats is accompanied by the oxidative destruction of some of the fat-soluble vitamins naturally or accidentally associated with them. Hopkins (3) was the first to demonstrate the rapid destruction of vitamin A by aëration and heat, a characteristic which later aided in the identification of vitamin D as a separate entity. Later Fridericia (4) reported that when certain animal fats, such as lard or hydrogenated whale oil, were mixed in a ration containing butter as the source of vitamin A, animals were no longer protected from vitamin A deficiency. He believed the destruction of vitamin A came about through the oxidizing action of peroxides formed when the unsaturated fats absorbed oxygen. The vegetable fats examined had no such effect.

* A part time grant from the National Live Stock and Meat Board is gratefully acknowledged.

Powick (5) reported similar results, and the experiences of McCollum, Simmonds and Becker (6) with "salt ophthalmia" are of particular interest in that the offending salt was finally shown to be ferrous sulfate whose presence greatly accelerates the oxidation of unsaturated fats (7, 8, 9).

Vitamin E is more sensitive than vitamin A to the destructive influence of this type of oxidation. The experiments of Mattill, Carman and Clayton (10) showed that a milk diet adequate for reproduction produced sterility when lard was added to it although no indication of vitamin A impairment was noted. Similar evidence can be drawn from other investigations of vitamin E in which highly desiccated rations or rations containing unsaturated animal fats have been used, the most recent being the work of Clayton (11). The most clear-cut demonstration of the specific vulnerability of vitamin E as compared with A has been given by Waddell and Steenbock (12). Several rations entirely adequate for growth and reproduction produced only sterile animals after a dilute ether solution of ferric chloride had been evaporated upon them, although they were still capable of curing ophthalmia.

To explain the destruction of vitamin E in mixtures containing unsaturated fats, Evans and Burr (13) assumed the presence of "anti-vitamins"; but Fridericia's explanation that peroxides were concerned in the disappearance of vitamin A is a more tangible basis for the loss of vitamin E under these conditions. Mattill (9) has pointed out that wheat germ and some other vegetable oils, because they contain more hydroxyl groups (as measured by their acetyl value) than do animal fats such as butter, lard, and cod liver oil, retard auto-oxidation and the accompanying inactivation of vitamins A and E. More recently (14) it has been shown that certain of the sterols present in corn oil are effective in delaying auto-oxidation, and that their effectiveness is destroyed by acetylation. Such retarding agents may be called anti-oxidants, or more preferably (15) anti-oxygens.

In view of this apparent relation between auto-oxidation of fats and sterility, it seemed desirable to compare the reproductive behavior of animals on diets containing various fats with the susceptibility of those fats to oxidation. This paper deals with a chemical study of the oxidation of such fats and with observations on the animals reared on rations containing them.

Experimental

The rations employed consisted of casein (18 per cent), starch (49 per cent) salts[1] (4 per cent), yeast[2] (5 per cent), and a fat mixture (24 per cent). In one case (Ration 230) the proportions were slightly different; the fat mixtures are given in Table I.

TABLE I

Ration No.	Fat Mixture
225	Cottonseed oil 15, butter 9
226	Cottonseed oil 22, cod liver oil 2 (added daily)
227	Hydrogenated vegetable fat* 15, butter 9
228	Hydrogenated vegetable fat* 22, cod liver oil 2 (added daily)
229	Hydrogenated lard 15, butter 9
230	Lard 17, cod liver oil 2

The butter fat was obtained from a good grade of creamery butter freed from water, salt and curd. The cottonseed oil was a market product, as was the lard. The cod liver oil was Norwegian; the hydrogenated lard was prepared in the laboratory by means of a nickel catalyst furnished by the Procter and Gamble Co. The stearin later substituted for the hydrogenated lard was a commercial product of relatively poor grade. The wheat embryo oil later added to the rations was prepared by cold ether extraction of wheat germ. The rations were prepared usually in kilogram quantities and kept in open containers at the temperature of the animal room until fed.

A. Chemical Studies

Since the complete rations differed in consistency owing to the different melting points of the fats, it seemed advisable to study the oxidation of the fat mixtures apart from the other constituents of the ration, whose presence, furthermore, would interfere greatly with the tests proposed.

The susceptibility of the fats to oxidation was determined by the method of Greenbank and Holm (17). The automatic registration device described elsewhere (14) was unfortunately not employed throughout, and this device is a great convenience if, indeed, not an essential requirement for accurate results. The fats were weighed into 300 cc. Erleymeyer flasks. The flasks were placed in a bath at 70°C or 100°C and after displacement of the air by oxygen they were connected with open mercury manometers outside the bath. From time to time the pressure changes were recorded. The time

[1] The Osborne and Mendel salt mixture enriched with some of the rarer minerals as suggested by Daniels and Hutton (16).

[2] Supplied by the Fleischmann Company.

* Crisco

from the beginning of the heating to the first indication of oxygen absorption, designated as the induction period, was used as an index of auto-oxidizability, and data on the induction periods of the fats here studied are given in Table II. Despite great variability it is evident that the fats were auto-oxidizable with increasing slowness in the following order; cod liver

TABLE II

INDUCTION PERIODS OF AUTO-OXIDIZABLE FATS

Fat	Temperature	Number of tests	Range hours	Average hours
Cod liver oil	70° C	5	1–5	3
Lard	70	1*		< 1
Lard	70	10	5–15	10
Lard	70	10	18–35	27
Lard	70	3	50–75	63
Lard	100	15	1–4	2.5
Butter	70	3	6–15	12
Butter	70	6	20–39	30
Butter	70	1		50
Butter	70	1		94
Butter	100	6	2–6	4.5
Cottonseed oil	70	19	18–75	

* An old sample that had stood open at room temperature for several months.

oil, lard, butter fat, cottonseed oil. Hydrogenated vegetable fat* and stearin, being practically unauto-oxidizable under the conditions, are omitted from the table.

The great variability, not only between different lots of the same fat but also often between samples of the same lot under apparently uniform conditions, is very disturbing, and only in part explicable; but if reworked butter keeps less well when the outer layer is not first removed (18) it is perhaps not surprising that a seemingly uniform sample of fat should show different oxidation-induction periods. Equally wide variations in the induction periods of some of these fats are reported by Triebold (19). At 95°C three poor lard samples had induction periods of 0.25, 0.5 and 1 hour; three medium lards 0.5, 1.5, 2.75 hours, and three good lards 2.75, 4.00 and 4.75 hours. A sample of cottonseed oil gave a 1-hour induction period and three samples of compound of corn and cottonseed oils gave 0.25 hour, and one sample, 0.75 hour. In so far as it is possible to judge from these and our figures, the temperature coefficient of the induction-oxidation process is that of a chemical reaction. With scrupulous attention to cleanliness of glassware and uniformity of procedure, it has nevertheless been found

* Crisco.

desirable to carry a control sample or blank with each group of tests in which possible modifying influences are introduced.

When the fats were combined as in the diets, they tended mutually to influence the auto-oxidizability of the mixture. Thus, as an example, the induction period of a mixture of lard and cod liver oil (Ration 230) was approximately 9 hours. The presence of 2 grams of wheat germ oil in this mixture prolonged the induction period beyond 24 hours. Again, the induction period of cottonseed oil was reduced from 75 hours to 21 hours by the addition of 2 grams of cod liver oil. Cottonseed oil contains some antioxidant, as its induction period and unpublished observations indicate, but not a sufficient amount to counteract the pro-oxidizing activity of cod liver oil with the result that in combination both oils were oxidized, the one more slowly, the other more rapidly, than alone. When 2 grams of wheat germ oil were added to this combination the induction period was lengthened to 30 hours; the amount of antioxidant was still inadequate to compete with the oxidizing activity of cod liver oil. Wheat germ oil is itself auto-oxidizable, and when its anti-oxidant is exhausted its own oxidation increases the volume of oxygen consumed. According to Moureu and Dufraisse (20) anti-oxidants must be easily oxidizable substances although this has recently been contradicted by Milas (21). The mixture of stearin and butter had a shorter induction period than butter alone, from which it would appear that stearin, although itself not auto-oxidizable, contained pro-oxidant material.

A further interesting observation upon these fat mixtures was a measure of the water-soluble oxidizable substances present in the fats, both fresh and after exposure to oxygen. The method used was a modification of Issoglio's procedure for estimating the water-soluble oxidizable substances present in rancid fats, as described by Kerr and Sorber (22).

Weighed amounts of fat were digested with distilled water on a steam bath for two hours, filtered hot through a wet paper, and an aliquot of the aqueous filtrate was titrated with N/100 permanganate, the oxidizability value being the titration figure multiplied by 80 and divided by the weight in grams of the fat taken. This result must be corrected by a blank determination.

The results of this study are given in Table III along with the physical changes noted in the fats (color and odor), after exposure to oxygen. The auto-oxidizability of fats as given in Table II is not necessarily related to the oxidizability value of the fats as given in column 3, Table III. The latter depends upon the number and character of the double bonds and

TABLE III

Fat or Ration No.		Oxidizability value Fresh fat	Auto-oxidized fat	Color	Odor
		Mg.	Mg.		
1.	Cod liver oil	116.0	1228.0	gone	rancid
2.	Lard	4.8	57.6	—	rancid
3.	Butter	10.4	36.0		
			105.7	gone	rancid
4.	Cottonseed oil	1.4	78.4	gone	rancid
5.	Hydrogenated vegetable fat*	.53	6.4		
			11.8	—	sweet
6.	Stearin	0	32.0	—	—
7.	Ration 225, cottonseed (15) butter (9)	4.3	30.3	—	sl. rancid
8.	Same with WGO**	4.6	35.0	—	sl. rancid
9.	Ration 226, cottonseed (22) cod liver oil (2)	5.0	94.1	gone	rancid
10.	Same with WGO**	3.7	94.7	gone	rancid
11.	Ration 227, Hydrogenated vegetable fat* (15) butter (9)	5.3	12.3	—	—
12.	Same with WGO**	6.7	44.6	—	sl. rancid
13.	Ration 228, Hydrogenated vegetable fat* (22) cod liver oil (2)	5.3	120.7	—	sl. rancid
14.	Same with WGO**	5.8	91.7	—	sl. rancid
15.	Ration 229, stearin (15) butter (9)	2.6	33.6	—	rancid
16.	Ration 230, lard (17) cod liver oil (2)	7.	56.0	—	rancid
			186.9		rancid
17.	Same with WGO**	10.9	21.3	—	—
			136.8	—	sl. rancid

* Crisco

** Wheat germ oil (2%) in place of 2% of the major fat.

their oxidation products, the former upon the accelerating or inhibiting substances present. While both may be related to the oxidative destruction of vitamin E it is difficult to make any correlation because it was not possible to examine the oxidized fats at strictly comparable intervals. Some of the more slowly oxidizing fats were still absorbing oxygen after 90 hours, others had ceased absorbing at 50 hours. The few cases in which two samples of the oxidized fat or mixture were examined at different intervals (Nos. 3, 5, 16, 17) reveal the difficulty of this choice. The absorption of equal amounts of oxygen might be fixed as a basis of comparison and this aspect should be studied more fully. However, the formation of aldehydes, ketones and other oxidizable products does not necessarily parallel the rate of absorption of oxygen. Greenbank and Holm have pointed out (2) that the progress of oxidation is not the same in a fatty acid

containing one double bonded carbon as it is in one containing two of them. The induction period of lard was shorter than that of cottonseed oil, while the water-soluble oxidizable products of cottonseed oil were more abundant than those of lard. Hydrogenated vegetable fat and stearin were equally inactive in absorbing oxygen but after exposure to oxygen the latter contained unexpected amounts of water-soluble oxidizable products whose precursors probably account for the shortened induction period of a mixture of butter and stearin over that of butter alone. It is again apparent that the presence of wheat germ oil may delay auto-oxidation and the formation of oxidizable products, but when the exposure to oxygen and heat outlasts the retardation provided by its anti-oxidant, even larger amounts of oxidizable products may be formed than would be in the absence of wheat germ oil.

The changes in odor and color of the oxidized fats together with the results of occasional Kreis and peroxide tests were in agreement with the passage of the induction period, and the duration of the subsequent period of oxidation, although the Kreis test has recently been judged unsatisfactory as a criterion of rancidity (23).

If the efficacy of a ration for reproduction were to be determined by the ability of its contained fats to resist oxidation with attendant disappearance of the vitamin E present, the most efficient combination examined as judged by these chemical studies, should be that of hydrogenated vegetable oil with butter (Ration 227). Cottonseed oil with butter should be less so (Ration 225) and the replacement of butter by cod liver oil in these diets would impair both of them (Rations 228 and 226). The rapid oxidation of butter in the presence of stearin would throw some doubt on the value of this combination (Ration 229) and the combination of lard and cod liver oil should be least satisfactory (Ration 230). This statement takes no account of the presence or absence of vitamin E in these fats *per se*. Data on the distribution of vitamin E have not been obtained apart from possible association with auto-oxidizable fats, peroxides or anti-oxidants.

B. Animal Experiments

The rats used in these tests were from our stock colony. About half the animals on each ration were six weeks old, weighing 100 to 125 grams, the others were four weeks old weighing about 50 grams. This differentiation was made in order to reveal the influence, if any, of body stores. The rats grew to be sleek and well nourished in nearly every case and at autopsy only three cases of lung infection were found in the entire series of 95

animals. The data on reproduction are briefly summarized in Table IV.

The hydrogenated vegetable oil-butter ration (227) was most successful, in that second generation animals were fertile, while the stearin-butter (229) and the lard-cod liver oil (230) rations were least successful, causing

TABLE IV

Ration Numbers and fat content*	Males Fert.	Males Ster.	Females	Av. No. litters per animal	Av. No. young in litter	Av. No. resorp's.	Remarks
225; Cotton seed (15) butter (9)**	6	0	8	1.9	5.2?	0.6	Two litters not seen
226; Cottonseed (22) cod liver oil (2) (daily)**	7	0	7	1.9	6.3	0.4	1 female pregnant at autopsy
2nd generation	2	0	1	0	—	—	2nd generation obese
227; Hydrog. veg. fat† (15) butter (9)**	9	0	8	1.6	4.6	0	1 female pregnant at autopsy
2nd generation	1	0	2	2.5	6.6	0	1 female pregnant at autopsy
228; Hydrog. veg. fat†(22) cod liver oil (2) (daily)**	9	0	8	2.4	4.7	0	
229; Hydrog. lard (stearin) (15) butter (9)	2	3	7	1.3	4.1	0	Testes weights of sterile males 46–61% of the normal
230; Lard (17) cod liver oil (2)	2	5	8	0.4	3.7	0.75	

* Figures in parenthesis are per cent of the fats in the basal ration.

** During the progress of the experiment these rations were reinforced by 2% of wheat germ oil in place of 2% of the principal fat, but with little or no change in the reproductive behavior of the animals.

† Crisco.

resorptions and sterile males. The cottonseed rations, with butter (225) and with cod liver oil (226) gave somewhat comparable performances in showing resorptions. The hydrogenated vegetable oil-cod liver oil ration (228) maintained fertility but no young were reared.

An unfair advantage was unfortunately conferred upon the rations containing cod liver oil by its daily incorporation into the ration instead of at longer intervals. Its destructive oxidizing effect was therefore exerted over a period of only 24 hours and at room temperature, in the presence of the anti-oxidants in cottonseed oil and hydrogenated vegetable oil. In view of

the probable temperature coefficient of the reaction expressed by the induction period, little if any of the vitamin E was destroyed under these conditions, unless we assume that oxidation processes are influenced by these factors within the body as well as in the ration. Evidence on this point is greatly needed, especially since it will lead us toward an understanding of the functions of the fat-soluble vitamins. An addition of wheat germ oil equivalent to 2 per cent of the ration had little or no effect in modifying the reproductive behavior. There were resorptions on rations 225 and 226 both before and after the introduction of this oil and rations 227 and 228 gave no indication of inadequacy without such addition. These facts are in agreement with the observations on induction periods and oxidizable products of the fat mixtures. Previous experience has shown (24) that synthetic sterility-producing rations containing small amounts of lard (3 per cent) may be improved by the addition of as little as 1 and 2 per cent of wheat germ oil, pregnancies in the majority of animals being secured after 9 and 6 weeks respectively. Four per cent additions of wheat germ oil to such rations produced pregnancies in less than a week and in a greater proportion of animals. The presence of larger amounts of lard (22 per cent) greatly decreased the efficiency of the added wheat germ oil. More extensive data of Evans and Burr (13) on this point confirm the interpretation that 2 per cent of wheat germ oil can not be expected to exert any great anti-oxidant effect in the rations here studied.*

Objection may be raised to the emphasis placed upon auto-oxidation and to the neglect of the original vitamin E content of the fats employed. The answer to this objection is that all of the diets used contained amounts of vitamin E which under proper conditions have been found adequate.

The only ration which may be questioned on this score is the one containing lard and cod liver oil (Ration 230). Contrary to most observations are those of Simmonds, Becker and McCollum (25) and of Nelson and his coworkers (26) which demonstrate that lard and cod liver oil samples may at times contain sufficient vitamin E for continued reproduction. These two fats and butter fat were the fat constituents of the deficient rations which led to the discovery of vitamin E. Skim milk powder has also been used in basal sterility-producing diets, but under proper conditions relatively small amounts of skim milk powder supply enough vitamin E for successful reproduction (27, 28). Our knowledge of the occurrence of

* The marked superiority of wheat germ over wheat germ oil (24), for which there was no explanation at the time, is possibly related to the development of peroxides in ether repeatedly used for the extraction of wheat germ. Oil so obtained may give a positive peroxide test and its effectiveness as an anti-oxidant is correspondingly reduced.

vitamin E is thus relative, and not absolute. The conditions which determine its effectiveness include, as the only variable thus far identified, the agencies which make for or against auto-oxidation of the fats.

This aspect is brought out more clearly by the tests made with cottonseed oil and hydrogenated cottonseed oil (Rations 225 to 228). The original observations of Evans and Burr (29) on these two fats indicate that the latter is superior to cottonseed oil for reproduction. It is hardly to be supposed that hydrogenation produces vitamin E (apparently it is not destroyed by this process) and therefore the product cannot well possess more vitamin E than the oil from which it is made. Unpublished observations indicate that pro-oxygenic substances can be removed from oils by adsorption; among the final steps in the manufacture of hydrogenated oils is a treatment with some adsorbent like Kieselguhr, with subsequent filtration. Hydrogenated cottonseed oil is a richer source of vitamin E than cottonseed oil because it is less readily auto-oxidized, having lost pro-oxidants and a large portion of its unsaturated fatty acids.

Further comparison of the hydrogenated and unhydrogenated cottonseed oil rations with reference to the accompanying fats, butter and cod-liver oil, leads to less definite conclusions, since, in the case of the hydrogenated the use of cod-liver oil (daily) instead of butter fat produced a definitely inferior ration (Table IV). In the case of cottonseed oil this difference in the accompanying fat was slightly but perhaps not significantly in favor of cod-liver oil.

The diet containing hydrogenated lard (stearin) and butter caused degenerated testes but no resorptions, from which it might be concluded that male animals are more sensitive to the lack of vitamin E than female animals. The opposite condition is the more probable as indicated by the behavior of the animals on the other diets. The variability in reproductive behavior is too great to permit any positive statement on this point from the small number of animals used. One statement can be made with certainty, on the basis of the reduced induction period of the stearin butter mixture and the water-soluble oxidation products of stearin after exposure to oxygen—namely that conditions were favorable for the auto-oxidative destruction of vitamin E. The males which went on this diet at six weeks of age, with one exception, retained their fertility longer than those which went on it at four weeks of age and the same is true of the animals, both male and female, on diet 230. No such correlation is possible with the female animals on the other rations.

Several interesting questions are suggested by the association of vitamin E in nature with materials of anti-oxygenic capacity, and by the ease with

which it is destroyed in the presence of pro-oxygenic substances. It may be that the physiological activity of vitamin E resides in the anti-oxygenic property, and the identity of anti-oxidant and vitamin E is favored by the single observation of Evans and Burr (29) on the inadequacy of a potent concentrate to evoke fertility after acetylation. The loss of anti-oxidant power through acetylation has been demonstrated not only with corn oil (14) but also with sterols from other natural sources and with substances of known composition (unpublished). From several papers it is clear that vitamin E-rich materials protect vitamin A in mixed rations and a typical anti-oxidant like hydroquinone (30, 31) protects both A and D. The successful administration of vitamin E concentrates apart from the basal ration (32, 33) indicates that the function of vitamin E is more than that of a preservative, unless, by its specific anti-oxidizing capacity, it controls the progress of oxidation in the tissues. The fact that substances containing vitamin E seem to possess growth-promoting capacity apart from the fertility-conferring power (33, 34) may mean that one of the functions of vitamin E within the body is to accomplish a more economical use of the other fat-soluble vitamins, and the inability of an anti-oxidant like hydroquinone to replace vitamin A in the diet (35) is not inconsistent with this hypothesis. The recent conclusion of Mason (36) to the effect that vitamins A and E are jointly concerned in the maintenance of fertility in male animals lends further weight to this argument.

Progress in unraveling this skein of complicated relationships can come only with the separation of the individual strands, the preparation of purer starting materials, and a study of their individual chemical and physiological properties.

Summary

Observations on the susceptibility to oxidation of several fats and oils have been correlated with the reproductive behavior of rats reared on food mixtures containing these same fats. The auto-oxidizability of the fats was measured by the induction period of oxygen absorption and the length of this period increased in the following order; cod liver oil, lard, butter fat, cottonseed oil, stearin, hydrogenated cottonseed oil. When mixed as in the diets, the fats mutually influenced the auto-oxidizability of the mixture. The reproductive behavior of animals, as indicated by the numbers of litters, the frequency of resorptions and the weights of the testes, indicated that the mixture of hydrogenated cottonseed oil and butter was most satisfactory, that of lard and cod liver oil and that of stearin and butter (which had an unexpectedly short induction period) were least successful. Intermediate were the mixtures of cottonseed oil with butter and with cod

liver oil, and the combination of hydrogenated cottonseed oil with cod liver oil. The animal tests are in fairly good agreement with the chemical tests and indicate that our knowledge of the distribution of vitamin E is relative rather than absolute. The efficiency of a given source of vitamin E depends in part upon the auto-oxidizable materials and anti-oxidants which are associated with it. The possible rôle of vitamin E as an anti-oxidant is discussed.

BIBLIOGRAPHY

1. Greenbank, G. R., and Holm, G. E., *Ind. and Eng. Chem.* 1924, XVI, 598.
2. Holm, G. E., Greenbank, G. R., and Deysher, E. F., *Ind. and Eng. Chem.*, 1927, XIX, 156.
3. Hopkins, F. G., *Biochem. Jour.*, 1920, XIV, 725.
4. Fridericia, L. S., *Jour. Biol. Chem.*, 1924, LXII, 471.
5. Powick, W. E., *Jour. Agri. Research*, 1925, XXXI, 1017.
6. McCollum, E. V., Simmonds, N., and Becker, J. E., *Jour. Biol. Chem.*, 1925, LXIV, 161.
7. Jones, J. H., *Jour. Biol. Chem.*, 1927, LXXV, 139.
8. McCollum, E. V., Simmonds, N., and Becker, J. E., *Proc. Soc. Exp. Biol. and Med.*, 1927, XXIV, 952.
9. Mattill, H. A., *Jour. Amer. Med. Assoc.*, 1927, LXXXIX, 1505.
10. Mattill, H. A., Carman, J. S., and Clayton, M. M., *Jour. Biol. Chem.*, 1924, LXI, 729.
11. Clayton, M. M., *This Jour.*, 1930. II, 491.
12. Waddell, J., and Steenbock, H., *Jour. Biol. Chem.*, 1928, LXXX, 431.
13. Evans, H. M., and Burr, G. O., *Jour. Amer. Med. Assoc.*, 1927, LXXXIX, 1587.
14. Mattill, H. A., and Crawford, B., *Ind. and Eng. Chem.*, 1930, XXII, 341.
15. Moureu, C., Dufraisse, C., and Lotte, P., *Ind. and Eng. Chem.*, 1930, XXII, 549.
16. Daniels, A. L., and Hutton, M. K., *Jour. Biol. Chem.*, 1925, LXIII, 143.
17. Greenbank, G. R., and Holm, G. E., *Ind. and Eng. Chem.* 1925, XVII, 625.
18. Gratz, O., Proc. 8th World's Dairy Congress, 1928, 456; through *Chem. Abs.*, 1929, XXIII, 5516.
19. Triebold, H., Thesis in Agricultural Biochemistry, University of Minnesota, 1926.
20. Moureu, C., and Dufraisse, C., *Chem. Rev.*, 1926, III, 113.
21. Milas, N. A., *Jour. Amer. Chem. Soc.*, 1930, LII, 739.
22. Kerr, R. H., and Sorber, D. G., *Ind. and Eng. Chem.*, 1925, XV, 383.
23. Richardson, A. S., et al., *Oil and Fat Ind.*, 1929, VI, 28; through *Chem. Abs.*, 1929, XXIII, 4359.
24. Mattill, H. A., and Clayton, M. M., *Jour. Biol. Chem.*, 1926, LXVIII, 665.
25. Simmonds, N., Becker, J. E., and McCollum, E. V., *This Jour.*, 1928, I, 29.
26. Nelson, V. E., Ohrbeck, E., Jones, R. L., and Taylor, M. W., *Amer. Jour. Physiol.*, 1928, LXXXV, 476.
27. Anderegg, L. T., and Nelson, V. E., *Ind. and Eng. Chem.*, 1926, XVIII, 620.
28. Clayton, M. M., *This Jour.*, 1930, III, 23.
29. Evans, H. M., and Burr, G. O., Memoirs, University of California, 1927, Volume 8.
30. Huston, R. C., and Lightbody, H. C., *Jour. Biol. Chem.*, 1928, LXXVI, 547.
31. Huston, R. C., Lightbody, H. C., and Ball, C. D. Jr., *Jour. Biol. Chem.*, 1928, LXXIX, 507.
32. Daniels, A. L., and Jordan, D. P., *Proc. Soc. Exp. Biol. and Med.*, 1928, XXVI, 185.
33. Evans, H. M., *This Jour.*, 1928, I, 23.
34. Mason, K. E., *This Jour.*, 1929, I, 311.
35. Beard, H. H., Pomerene, E., and Shipley, R. A., *Proc. Soc. Exp. Biol. and Med.*, 1929, XXVI, 328.
36. Mason, K. E., *Jour. Exp. Zoöl.*, 1930, LV, 101.

Editorial Review

THE ROLE OF DIET IN THE CAUSE, PREVENTION, AND CURE OF DENTAL DISEASES*

WHAT we know today about the origin of dental diseases such as dental caries, inflammation of the soft oral tissue and pyorrhea has been slowly accumulated over a period of nearly 2000 years. As is the case with development of any kind, the periods of progress, and of relapse, are intimately associated with ideas. I shall try, in this article, to trace the history of the ideas, and to discuss them in the light of our present knowledge in so far as I am capable of discussing them.

A rather popular misconception, that is entertained not only by the layman but also by many professional people, is that dental caries, gingival inflammation and pyorrhea are the more or less direct outcome of ingesting soft foods. The soft foods are said not to clean the teeth, to lodge between the teeth and to afford no mechanical stimulation to the gingival tissue. We further obtain the impression that this habit of eating soft food is a foible of the modern generation and that the incidence of dental diseases has been greatly increased by this modern practice.

Historical

A careful study of the chapter on "The Abnormalities and Pathology of Ancient Egyptian Teeth" in "Studies in the Palaeopathology of Egypt" by M. A. Ruffer, pages 268–321, University of Chicago Press, 1921, shows quite conclusively that mankind has been afflicted with dental caries and suppurative periodontitis as far back as records of any kind have been preserved. These conditions may be the outcome of civilization but they are certainly not the outcome of modern civilization.

The teeth of the early Egyptians show extensive attrition which was doubtless due to the coarse quality of the food. . . . . A few quotations from Ruffer's book, with regard to the early Egyptian diet, are very instructive.

"The Egyptians were nicknamed eaters of bread. . . . . It was made from spelt, bearded wheat or barley. . . . . Flour was prepared already in the predynastic period by grinding grain between two stones or pounding it in a mortar. . . . . The specimens of old Egyptian bread which I have examined consist of a very coarse paste usually containing a large amount of husks, and some straw even, together with coarse fragments of unbroken wheat or barley grain. Undoubtedly this bread did not improve the masticating surfaces even if it did contain plenty of fibrous material to clean the interstices of the teeth.

The teeth were not improved by the mastication of the roots of marshy plants, which, raw or cooked, the poorer Egyptians—and possibly the richer—ate in large quantities. Indeed, many of

* The author, Dr. Milton Theo. Hanke, is associate Professor of Biochemistry in the Department of Pathology at the University of Chicago and a member of the Otho S. A. Sprague Memorial Institute, Chicago, Illinois.—Ed.

the children of the poorer classes were brought up on them. The chief fruit eaten by the Egyptian consisted of grapes, figs, dates, pomegranates, melons, carobs, olives, apricots (?) and the seeds of marshy plants. . . . . Egypt has never been a great fruit growing country and the Egyptians of the poorer classes probably tasted little fruit except dates and melons.

"Attrition was intensified by the consumption of raw vegetables. Lentils, beans, artichokes, asparagus, beetroot and cabbages doubtless were cooked before being served up; but, on the other hand, onions, cucumbers, garlic, radishes, turnips etc. were eaten raw, with bread, as is done now." Animals and fish were also articles of food.

These quotations will suffice to show that the food was very coarse, that it consisted predominantly of carbohydrate and that it was very deficient in vitamins, particularly vitamin C. A deficiency in vitamin D could hardly have been a factor in Egypt and this statement is strengthened by the fact that rickets was almost unknown in this country.

"Neither syphilitic nor rickety teeth were discovered, and this observation confirms the evidence derived from the study of skeletons, for no syphilitic bone dating from ancient times in Egypt has been unearthed so far, and rachitis was very rare indeed, if it existed."

These studies of the conditions that existed in ancient Egypt are instructive because they show that:

1.—The mastication of coarse food does not prevent dental caries, suppurative periodontitis nor pyorrhea; but it does wear down the teeth.

2.—Unrefined grain does not contain mysterious substances that prevent dental disorders.

3.—These dental diseases were associated with a diet that was predominantly carbohydrate, that was probably markedly deficient in vitamin C and that was not necessarily inadequate in vitamin D.

4.—Dental diseases are certainly not the outcome of modern civilization.

We are now ready to consider the ideas that have served as guides in studying the causes of dental diseases. The first idea that is at all definite is that toothache is caused by the stagnation of depraved juices in the teeth. (Hippocrates, 456 B.C.). This idea recurs in 1732 A.D. when Krautermann (1) states "The teeth are corroded by the great influx of the *lymphae acrioris*. The *fermento acri rodenti* in the hollow tooth appears after being removed by the application of remedies."

A statement that sounds almost modern was made by Bourdet in 1757 (2). "When the juices contained in the vessels of the teeth are too thick, they stagnate, decay and soon attack the tooth." The experiments of Findlay (3) have shown that the syndrome called scurvy is primarily due to a swelling of the endothelium of the capillaries with consequent stagnation of the blood and improper aeration of the surrounding tissues. The work of Höjer (4), Zilva and Wells (5), and Howe (6) has clearly demonstrated that a deficiency in vitamin C produces changes in the pulps of a guinea pig's teeth before any change is demonstrable anywhere else in the body. A change is histologically demonstrable after the guinea pig has subsisted for 48 hours on a diet devoid of vitamin C. These investigators have demonstrated that a stagnation of the fluids in the tooth does occur, they have shown that this condition is produced by faulty diet and they have further shown that these changes are probably manifestations of scurvy.

That the teeth of scorbutic monkeys are prone to decay and conversely, that the teeth of well fed, non-scorbutic monkeys, do not decay, has been amply demonstrated by Howe (6). From this we may, at least tentatively, conclude that vitamin C (or a balanced diet) is a factor in preventing caries (and many other conditions as will be shown later) and that this tendency toward caries may, in some way, be associated with the changes that have occurred in the pulps of the teeth. The old idea of Hippocrates has, therefore, received considerable confirmation in modern times.

Galen, in 131 A.D., made some interesting observations on the relation of food to the incidence of oral pathology. He says "The lack of nutrition makes the teeth weak, thin and brittle. An excess of nutrition excites a kind of inflammation similar to that of the soft parts. A deficiency of

nourishment not only causes the tooth to die away, but also enlarges the cavities." Loosening of the teeth was said to be caused "by an excess of moisture which impairs the nerves."

We have here a number of ideas that have been the subject of considerable subsequent argument and experimentation, namely:

1.—Starvation leads to soft, brittle teeth. (We would probably say imperfect calcification.)

2.—Excessive nourishment, which probably meant the over ingestion of carbohydrate to the exclusion of fruits and vegetables (as it does today), produces toothache, caries and an inflammation of the soft oral tissue. We have here the first suggestion of the inflammation theory of caries (which is not so different from the idea expressed by Hippocrates) and the very interesting observation that "inflammation of the tooth" was frequently associated with inflammation of the soft oral tissue.

3.—The idea that the loosening of the teeth is due to an edema; which is, possibly, the correct explanation.

The idea that improper nutrition is a cause of dental diseases will be discussed in detail later. We wish to consider briefly the inflammation theory as applied to dental caries. This idea, vaguely expressed by Galen in 131 A.D. has been advocated repeatedly by many investigators even up to recent times. The Köcker (Principles of Dental Surgery, page 111 (1842)) says "Caries, in fact, is that state of the tooth in which mortification has taken place in one part and inflammation in the part contiguous to it, the former originally produced by the latter, and the latter continually kept up by the former."

The inflammation theory received support and embellishment from Frank Abbott (7), Heitzmann and Boedecker (8). These investigators attempted to show that the changes that occurred in a carious tooth were similar to those that were produced in other body tissue by an inflammatory process. This attempt was not entirely successful and the unique structure of a tooth rather precludes the possibility of comparing it with any other structure in the body. Miller (9) strenuously opposed this idea; but that deep-seated changes are present in a tooth that contains a carious lesion was a generally accepted belief and is not difficult to demonstrate histologically. The heated arguments fomented largely around the question of how these changes are produced.

All of the above concepts have at least one thing in common. The changes observed in the teeth, the supporting structures and the gingival tissue are considered to be metabolic in origin and not due to some local disturbance. The idea that oral pathology is due to some local condition has, apparently, also been advocated at intervals by isolated investigators. Thus Paul of Aegina (636) said, "to preserve the teeth, take precautions against the spoiling of food in the stomach since the frequent vomiting resulting from it is very injurious to the teeth." Pasch (1767), Bücking (1782), Becker (1808) and Ringlemann (1824) believed that the teeth are injured by sour food and acids. Robertson (1835) said that the dissolution of food particles in the mouth produced acids which, in turn, decomposed the teeth. Rognard (1838) amplified the above idea by stating that sulphuric and nitric acid are liberated when the food particles are disintegrated. Tomes (1873) concludes that "caries is the effect of external causes in which so called 'vital' forces play *no* part; that it is due to the solvent action of acids which have been generated by fermentation going on in the mouth, the buccal mucus probably playing no small part in the matter; and when once the disintegration is established in some congenitally defective point, the accumulations of food and secretions in the oral cavity will intensify the mischief by furnishing new supplies of acids."

The outstanding champion of the idea that dental caries is primarily caused by the slow action of organic acids upon the inorganic constituents of the teeth, was Willoughby D. Miller. His forceful presentation, both in the scientific literature and in the finest book that has ever been written on the subject (9), have had a profound influence upon scientific thought and has been a stimulus to research for the past fifty years.

Miller believed that bacteria (many bacteria) produced lactic acid by catabolizing certain

carbohydrate substrates in the mouth. Starch was considered to be the chief offender because, being insoluble, the starch particles lodged between the teeth and at the gingival margins and thus afforded a foothold for the maintained local production of lactic acid by the bacteria. Miller did not deny the then prevalent belief that sugar might be of some importance but he reasoned that sugar is too soluble to remain in contact with the teeth for a sufficient period of time to be considered a likely source of lactic acid. Miller says (9) "I consider starch and amylaceous substances more detrimental to the teeth than sugar, particularly as sugar, being readily soluble, is soon carried away or so diluted with saliva as to be rendered harmless, whereas amylaceous matter adheres to the teeth for a greater length of time and consequently manifests a more continued action than sugar."

Miller believed that acid dissolves the inorganic salts (chiefly calcium phosphate) and that organic cementing material (albuminoids) is attacked by the exogenous proteolytic enzymes (secreted by the micro-organisms) and dissolved. The result of this dual activity is a cavity in the tooth.

According to this conception, dental caries is the end result of two chemical processes. The phenomenon is supposed to be identical for both living and dead teeth. The only way that dental caries can conceivably be arrested or prevented is to keep the teeth scrupulously clean, i.e., free from bacteria or free from carbohydrate.

Before I attempt to present the newer knowledge of this subject or to correlate the facts and ideas that have just been presented, it will probably be best to recall, briefly, the anatomical and chemical structures with which we have to deal and to summarize the facts.

Structure of Teeth

Four structures have to be considered in dental research (10):

1.—*Enamel.* This is the hardest structure in the human body, and consists, chemically, of approximately 0.4% of keratin and 95–97% of inorganic salts, predominantly calcium phosphate (22), the balance being volatile matter, probably water.

The fully formed enamel consists of hexagonal prisms that are held together by a very narrow band of cementing material. The body of the prisms appears to consist entirely of inorganic salts. The small amount of organic matter that can be found in enamel appears to be contained exclusively in the narrow band that makes the dividing line between the prisms. When a thin cross section of the enamel is treated with 1% hydrochloric acid, the central portion of the prisms dissolves readily and completely, thus suggesting that this portion of the prism is devoid of organic matter (11). The marginal zones are, of course, also decalcified; but there is left behind a network of insoluble organic matter. The marginal zones (cementing substance) are rapidly decalcified when a drop of 0.1 N.HCl is brought in contact with a thin section of enamel. The rods are left almost or quite unattacked (25). That this compact structure could contain living matter seems entirely unlikely (22), but certain investigators

have and are still maintaining that the enamel does contain living matter (12). I am quite convinced that substances may diffuse into and *through* the enamel from either side. This can, of course, occur in the absence of actual living matter.

2.—*Dentine* contains about 28% of organic matter (collagen and elastin), 10% of water and 62% of inorganic salts, predominantly calcium phosphate. Dentine is a microscopically porous structure. It is full of tubules that are wide at the pulp side and become quite narrow as they approach the enamel or the cementum. These tubules extend to the dento-enamel junction in the crown of the teeth and to the dento-cemental junction in the root of the tooth. The matrix between the tubules is heavily calcified. The tubules are apparently lined with a highly resistant membrane of protein (Neumann's sheath) and *they contain living protoplasm*, offshoots from the odontoblasts. That the dentine contains living matter has not ever, I believe, been seriously doubted and the development of histological technique soon gave graphic evidence that the tubules are filled with soft organic matter. J. Tomes was probably the first to demonstrate that the fibrils are a part of the odontoblasts (13). Some investigators have persistently maintained that the fibrils continue out into the enamel.

The dentine certainly contains living protoplasm, it is rich in albuminoids and contains less inorganic matter than does enamel and it contains many tubules that are sufficiently large to accommodate many micro-organisms.

3.—*Cementum* is essentially like bone. It is deposited upon the exterior surface of the dentine and thus converts the tooth into an essentially bone like structure. The deposition of cementum upon the root of a tooth acts as a stimulus for the formation of alveolar bone. Connective tissue fibres attach the cementum to the alveolar bone through the highly vascular layer of soft tissue that is called the periodontal membrane. Cementum is, apparently, capable of constant construction and destruction. It can be absorbed and it can be redeposited. It is less active, in this respect, than is the alveolar bone because orthodontic procedures usually lead to an absorption of alveolar bone, in the area of stress, without producing an absorption of the cementum. Cementum is, of course, much softer than either dentine or enamel. It contains less inorganic matter and is better nourished.

4.—The *pulp*, *periodontal membrane* and the *gingival tissue* can well be considered together because they are one tissue, embryologically, and they

have a common blood supply. We would expect, then, that a pathological change of metabolic origin that might occur in any one of these structures should show some change in the other two.

The pulp, beside containing blood vessels and nerves, is characterized by the presence of a compact layer of odontoblasts that lie immediately against the inner wall of the dentine. The fibrils that extend into the dentine are offshoots from the odontoblasts. The pulp is a highly vascular tissue. The odontoblasts are, normally, well nourished. An exchange of metabolism products between the odontoblast and the blood vessels of the pulp can occur easily and rapidly. This also insures a fairly efficient exchange of materials in the dentine as will be discussed later.

The periodontal membrane is a highly vascular tissue that is full of noncalcified fibrous tissue. The apical end of this membrane is nourished by blood vessels from below. These also supply the pulp. The main vascular supply is derived from the narrow spaces that border on the alveolar bone.

The gingival tissue, the periodontal membrane and the pulp are essentially one tissue. Differences in response that these tissues show toward metabolic disturbances will be occasioned by the size of the blood vessels, the environment, the strain to which these tissues are subjected and the possibility for infection.

Pathology of Teeth

The literature pertaining to dental pathology is a chaos of facts and fiction (the latter being the outcome of a hyperenthusiastic championship of a fixed idea) in which respect it is identical with the literature on any subject that has attracted attention for a long time. A few facts can be culled from this maze of misinformation which give some very definite clues as to the real cause of dental diseases. I have elected to discuss these under four captions:

1.—*Defects in the histological structure of teeth*

Poorly constructed teeth, delayed and irregular dentition, and slow development of the jaw bones frequently occur in children that have rickets. Rickets is, moreover, frequently associated with frank scurvy and it is more than likely that a rachitic child is also receiving an inadequate amount of vitamin C. Just how much of this defective structure can be attributed to vitamin C is not known. Entirely similar abnormalities of bone and tooth structure have been produced in rats and in dogs by feeding diets deficient only in vitamin D. We must, however, be very

cautious in transferring results from either of these animals to man because both the dog and the rat are almost insusceptible to a vitamin C deficiency.

It seems entirely logical that a defective tooth should become diseased more easily than one that is histologically perfect; and this is the view that is most generally held.

Hypoplasia of the enamel in the permanent teeth is rather common and such teeth usually decay readily; but some persons go through life without developing carious lesions in these defective teeth. I think we can safely conclude that to insure normal dentition and normal jaw bone development, the mother and the child must receive food that contains an adequate amount of vitamin D. That the diet must contain calcium and phosphorous in adequate amounts follows as a natural corollary.

2.—Caries of the enamel

The presence of white opaque areas on the teeth, especially along the gingival margin, is a very common occurrence (I am not refering here to the so-called mottled enamel). These areas are, at first, quite hard. The surfaces are smooth and shiny. The areas can not be penetrated with a probe at first; but, as time goes by they may become soft, and they may eventually become a carious lesion. If diet can have any restorative action on carious enamel, one would expect to obtain the best results in these cases. We have, indeed, had a few cases in which the white opaque areas have, apparently, become smaller or in which they seem to have disappeared; but these areas have shown *no* demonstrable change in most of our cases after a year of dietary management.

This opacity, which is undoubtedly a precursor to a certain kind of decay, is due to some change in the structure of the enamel rods. Investigators appear to agree that the change is one that involves the cementing substance between the rods (14). They usually attribute this change to a solution of the cementing substance by acids derived either from the saliva or from the action of bacteria upon food particles.

These white opaque areas are not, as is frequently supposed, necessarily associated with plaque deposits on the teeth. They can be found on teeth that have been kept clean and in areas where the revealing stain (16) does not produce a discoloration.

The claim has been made that even the soft variety of opaque area can be solidified and the original translucency be restored by immersing the extracted tooth in a solution of calcium salts (saliva or some other cal-

cium salt solution). This has been hesitatingly claimed by a number of investigators (14) and it is probably true. The explanation is, probably, that a crystallization of calcium salts occurs upon the crystals of which the enamel rods are composed. The opacity would, of course, disappear as soon as the air spaces between the enamel rods became filled with calcium salts. This might not restore the enamel to its original composition, because the organic matrix can not be replaced, but it would remove the opacity and might, indeed, produce a superior form of enamel. It is quite conceivable that this type of repair process actually does occur in the mouth due to some change in the character of the saliva.

Most of the enamel on human teeth is quite dense. There is no space between the enamel rods and hence no place in which bacteria could lodge. Practically every human tooth is, however, full of easily visible fissures. A microscopic examination of enamel rarely fails to reveal imperfections in structure such as tiny superficial cracks. Ground sections of enamel show that these cracks and fissures contain numerous micro-organisms; but the enamel may be quite free from decay.

Carious enamel is soft and consists of the debris of the enamel rods. Large pieces of the sharply outlined rods can be seen. It appears as if the cementing material has been liquefied thus liberating the rods.

3.—Caries of the Dentine

The carious process leads to a rapid disintegration of the dentine. Dentine "decays" far more rapidly than does enamel. The carious process removes both of the constituents of the dentine, namely, the calcium salts and the organic matter. The dark, leathery mass that is so frequently found in a carious lesion consists almost entirely of organic matter and this is, presumably, partially hydrolyzed albuminoid. This proves that the inorganic matter is lost more easily than is the organic matter. Caries of the dentine is usually considered to be identical with caries of the enamel, i.e., due to the action of dilute acids.

Many bacteria are known to produce an exogenous proteolytic enzyme capable of liquefying gelatin and of hydrolyzing soluble and insoluble proteins. This is not a property that is peculiar to bacteria. Such enzymes are found rather generally associated with living tissue. Enzymes may be derived from the bacteria in the mouth (15) (*not necessarily on the teeth*) or they may come from the cells of the soft oral tissue. Caries of the dentine might be at least partially due to the action of proteolytic enzymes on the organic matter of this structure. This concept is subject to the criti-

cism that we know of no case in which proteolytic enzymes attack a structure that is alive. We would be forced to assume, then, that the tooth or certain parts of the tooth are dead or certainly at a low ebb of existence before the structure could be attacked by proteolytic enzymes.

The acid, or, let us say, the bacterial theory of decay fails to explain some things. It is true that bacteria are always present in carious dentine or enamel; but this is not surprising in view of the fact that saliva contains from five millions to fifty million bacteria per cubic centimeter. This certainly does not prove that the bacteria are primarily responsible for the caries. It is also true that blue litmus paper turns red when it is held, for some time, in contact with carious dentine; but litmus changes color at a pH of 7.0 and this indicator will turn red if it is placed in contact with most gingival tissue. No one has, to my knowledge, ever demonstrated that decaying dentine contains sufficient acidity to disintegrate the tooth structure.

Many bacteria catabolize carbohydrates so that acids are formed. The production of acidity in the mouth is, therefore, entirely probable; but the saliva is a buffered liquid and can neutralize considerable acidity. Miller (9) found many kinds of bacteria in the dentinal tubules. He did not believe that any one micro-organism is responsible for dental caries. Bunting and his coworkers, however, believe that *Bacillus acidophilus* is specifically responsible for dental caries (26).

We are told that starch particles that lodge between the teeth are good breeding grounds for bacteria and that the bacteria produce lactic acid from the starch. It is curious, in this connection, that the action of ptyalin on starch appears to have been overlooked. It would be impossible for fermentable starch to remain in the mouth for very long because it would be hydrolyzed into dextrin and maltose, both soluble products, that would soon be carried off by the saliva. It is also a well known fact that of two teeth that are in direct contact, the one will sometimes decay while the other does not. They have both been subjected to the same fermentation mixture and if dental caries is a purely chemical process, both teeth should have been acted upon. It is also well known that the most scrupulous care of the oral cavity is no insurance against dental caries.

4.—Scurvy

This disease, which is now known to be due to a lack of the antiscorbutic vitamin in the diet, has long been known to produce profound changes in the oral tissues. One of the earliest descriptions of this condition can be

found in "A Collection of Tracts, Surgical and Medical" by John Colbatch (1699). He appears also to be one of the first to have recognized that orange and lemon juice will cure this condition.

"I now come to treat of the disease of alkalies in the scurvy, that reigning distemper from which few are altogether free; and is by most accounted incurable, or at least for the most part proves so.

I have frequently been told by some seamen and surgeons, that have had long voyages at sea, especially towards China and the Indies, that of a hundred men in a ship, not two of them but have been almost eaten up with the scurvy, their skin squalid and full of blotches, their gums eaten away, and their teeth ready to drop out, pains and aches all over their bodies, and yet on their landing at Cadiz, or therebouts, where is plenty of oranges and lemons, and eating large quantities of them, in one fortnight's time at farthest scarce one has failed of being perfectly cured. This is not a relation of one or two persons only, but what is generally agreed upon, and allowed by all to be truth."

Scurvy is now known to be essentially a capillary disease. According to Findlay (3) the essential pathological change in experimental scurvy is the swelling and degeneration of the capillary endothelium as a result of which the flow of blood through the capillaries is retarded and extreme congestion results. This degeneration of the endothelium results in damage to the intercellular substance as a consequence of which edema and hemorrhage occur, while the stagnation of blood results in deficient oxygenation of the tissues. It is not surprising, then, that capillary changes may occur in certain parts of the body thus producing certain kinds of local pathology even though the grosser changes, that are found in frank scurvy, are not demonstrable.

A few quotations from the excellent paper by J. Axel Höjer (4) summarize much of our present knowledge with regard to this condition.

"Scurvy reduces the nutritional state and the active function of probably all the most active, most rapidly growing and highest differentiated cells of the body. This reduction begins with the absence of antiscorbutic in the food. It becomes manifest at a rate that varies for the different cells and tissues; first in the odontoblasts, then in osteoblasts, muscle cells, lymphoid tissue, fibroblast, and salivary glands, then in the liver, adrenal, chondroblasts and last in the epithelium of the kidney. The order in which the changes set in and continue in odontoblasts, osteoblasts and chondroblasts seems to argue for the notion that phylogenetically older and histo-functionally less differentiated cells are later and more lightly struck by the deficiency of antiscorbutic" . . . . .
"The interpretation of scurvy as a general, though not uniform, atrophy of the different tissues and organs of the body agrees well with the clinical symptoms. I have not found any symptom, the scorbutic nature of which is probable, that does not receive its natural explanation through such an interpretation. Among such symptoms may be mentioned:

1.—The sub- or anacidity in the gastric juice (Bierich).

2.—The diminished adrenalin contents in the adrenal (McCarrison).

3.—The failing regeneration of white and red blood corpuscles, and their extraordinarily rapid new formation in healing (Solle and Rosenberg).

4.—The diminishing of the opsonic index (Fortunato).

5.—The diminished resistance to infection.

6.—The appearance of hemorrhages in complications during the convalescence."

The changes produced in guinea pig's teeth are summarized, by Höjer, in the following manner:

"1.—The gradual change of the odontoblast layer. This seems to be a sure sign of scurvy and one of the earliest changes to set in. The odontoblasts assume another shape, grow shorter, more rounded, and show another arrangement, the normal regular, creeper-like formation makes way to a layer, which in some places curves in towards the center of the pulp and is soon spilt through the cells secreting a hard tissue between themselves, so that the syncytial character of the cell layer does not plainly appear. The odontoblasts have been transformed into osteoblasts placed in bone canals. Incidentally may be pointed out how well this process agrees with the conception of the syncytial character of bone which Professor G. Häggquist has lately expressed in the Society of Swedish Physicians.

2.—The amorphous calcification of the predentine.

3.—Widening of Tomes' canals in the dentine formed before the onset of scurvy.

4.—New formation of bone instead of dentine. This bone is first lying as a thin layer inside the calcified predentine, but soon extends reticularly towards the center of the pulp. This bone has a spongy, porous character.

5.—Dilatation of vessels and in early stages hyperemia; sometimes hemorrhages in the pulp.

6.—Atrophy and resorption of pulpa tissue, pulpa cells, the newly formed bone and the old dentine. This resorption, which, as everywhere in bone formation, will surely be found also with scurvy in all stages, appears more distinctly after all the new bone formation in the final stage has stopped, and may proceed so far, that in place of pulpa tissue there is seen nothing but some large hollows filled with fluid.

7.—In the healing of the scurvy—at an earlier stage—reorganization of the pulpa bone into irregular dentine, osteodentine, with bone canals and dentinal canals.

8.—In a scurvy which is latent all through or very much mitigated, when at least half the amount of antiscorbutic needful to an individual is provided—forms which are the most common in man—the progress is similar, though not so pronounced and presents pictures, that differ considerably less from the normal. If the antiscorbutic dose provided is 0.5–0.7 of the minimum protective dose, an irregular dentine is formed with Tomes' canals in most places, but in the lingual part of the pulp there are found in some symmetric places ridges of a hard tissue, with the character of bone and lacking dentinal canals. In this pulpa bone as well as in the newly formed irregular dentine, there are canals of Havers' type and isolated bone corpuscles which may be considered to consist of transformed odontoblasts.

With an antiscorbutic dose of 0.8 or more of the minimum protective dose, the picture here described is changed so far, that there is no pulpa bone, but all the newly formed hard tissue in the pulpa consists of osteodentine, or even dentine (the first formed layer)."

Höjer also calls attention to the fact that the bone that is formed in scorbutic animals is not normal bone. The imperfectly formed matrix becomes properly calcified. The difficulty lies in the formation of the matrix.

Höjer was working primarily with guinea pigs; but his paper contains a correlation between the observations on this animal, other animals and man. The fundamental changes are much the same in all animals excepting of course, those that show a pronounced insusceptibility to a lack of

vitamin C, such as the rat. An observation of Eddy (personal communication) is of interest in this connection. He found that a disorganization of the odontoblasts occurred even in the rat when this animal was kept for some time on a diet devoid of vitamin C.

Howe (6) has been able to produce, in monkeys, all of the dental diseases with which the dentist comes in direct contact by feeding diets deficient solely in vitamin C.

It is hardly possible to make a systematic study of the changes in the structure of the pulps of human teeth that are associated with changes in nutrition. It is possible, however, to study changes in the thickness of the periodontal membrane by means of dental roentgenograms; and the quality of the gingival tissue can be determined by simple inspection, preferably with a biobjective, binocular microscope, in the living subject.

We (17) have been making such studies and have found that a diet rich in vitamin (particularly rich in vitamin C) will, not infrequently, reduce the apparent thickness of the periodontal membrane and will convert spongy or soft gingival tissue (both being hyperemic) into perfectly healthy gingival tissue. We have come, therefore, to consider these pathological conditions as being due to a lack of vitamin in the diet and we believe that these conditions are, more particularly, manifestations of scurvy. The pulp, the periodontal membrane and the gingival tissue are histologically continuous and embryologically derived from a common source. It is fair to conclude, therefore, that changes in the pulp may also occur in the human subject even prior to the changes that can be observed in the periodontal tissue and in the gingival tissue.

These considerations justify the conclusion that a deficiency in vitamin C may be a very important factor in producing pathological changes in the pulp, in the periodontal tissue and in the gingival tissue. This immediately throws a new light upon dental diseases and releases us from the shackles of the rather hopeless outlook that dental caries is a purely chemical process occasioned by the products of bacterial fermentation and that spongy gingival tissue and pyorrhea are primarily due to the ravages of bacteria that can hardly be removed from the mouth. It lays the stress upon the metabolism of body cells. If we can produce healthy cells, by proper nourishment, we can often eliminate the bacteria from the infected areas and keep them outside of the confines of the tissue.

Newer Ideas Regarding Dental Disease

The easily recognized pyorrheas are far from being a clinical entity. The simplest variety is due, merely, to a local irritation produced by sub-

gingival calculus. The condition disappears as soon as the calculus is removed and a satisfactory mouth hygiene has been established regardless of the diet. It is cases such as these that have furnished subject matter for many publications and have retarded our knowledge of the true cause of pyorrhea. Some kinds of pyorrhea have long been considered to be incurable because they could not be cured by simple scaling, by the use of local astringents or antiseptics and by proper mouth hygiene. Such cases can frequently be rendered free from pus and the loose teeth may even become more firm after surgical manipulation; but in time, two to three months, the patient returns for more treatment in spite of the most scrupulous kind of oral hygiene. Dental roentgenograms show a slow progressive loss of alveolar bone. These are true cases of pyorrhea and they may be of metabolic origin. We have studied such cases for two years and have found that, when the patient cooperates by eating the prescribed food, the progress of the disease stops (roentgenograms) and in most cases the teeth become tight and the pus disappears and does not recur. There are cases, of course, in which the bone destruction is so extensive that the teeth can not become tight. If the unsupported tooth is not subjected to much strain during the process of mastication, a change may occur such that the pus may not recur even though the tooth may remain quite loose. Under ordinary circumstances, however, such unsupported teeth must, eventually, be extracted. There are some cases that do not respond at all.

Some types of gingival inflammation (without pus) can be traced directly to a subgingival calculus, faulty dental work, traumatic occlusion or to the local irritation produced by bacteria. A correction of these conditions will, in these cases, frequently lead to a disappearance of the inflammation. There are, however, many cases of gingival inflammation that can not be traced to any of these causes.

An examination of such tissue, in the living subject, may show just a diffuse hyperemia with no marked increase in the size of the blood vessels and no particular thickening; or the tissue may be hyperemic with a border zone of engorged blood vessels and some thickening; or the tissue may be full of stagnated blood (even blue in color) with a broad zone of engorged blood vessels and a marked edema. This latter type of tissue (spongy) bleeds readily on the slightest provocation. Gentle pressure produces an exudation of bloody fluid and vigorous brushing with a stiff bristle tooth brush will tear off pieces of this tissue.

These gingival inflammations appear usually to be manifestations of malnutrition and they can be more particularly classed as phenomena of

scurvy. This conclusion is based upon the fact that when the afflicted persons are placed upon a diet that is rich in vitamins (particularly rich in vitamin C) the gingival tissue becomes healthy. Spongy tissue will usually stop bleeding in 14 to 30 days. Months may be required to produce perfectly healthy gingival tissue.

A vitamin rich diet will produce changes in the gingival tissue such that the tissue makes efforts to overcome even serious local irritants, as can be best illustrated by citing one case.

A woman, age 45, presented herself for treatment because she had pyorrhea. The gingival tissue was spongy, the teeth were loose and the roots were incrusted with subgingival calculus. X-rays were not obtained. The patient was given our usual dietary recommendations (18) and was not treated in any way. She returned three months later. The teeth were tight, the gingival tissue was healthy and the gums had receded below the calculus incrustations. The incrustations were removed. The gingival tissue slowly expanded upward to cover more of the cementum. This is, of course, a rare case. Pyorrhea can rarely be treated successfully without the aid of a dentist.

Many mouths become quite healthy even though the oral hygiene is poor or nil. Bacteria are, of course, a potent factor while the tissue is unhealthy; but healthy gingival tissue appears to remain healthy in some cases in spite of bacteria.

That dental caries can be arrested if the patient will eat the proper food seems probable from the investigations of Sherman Davis (19), Boyd, Drain and Nelson (20), Bunting, Hadley, Jay and Hard (21) and ourselves. All of us have been advocating about the same food. We differ some in that Davis, and Boyd and Drain have been feeding cod liver oil whereas we have adhered rather conscientiously to eggs, and in that we have been advocating the juice of one lemon and a pint of orange juice a day whereas the other investigators have given just eight ounces of orange juice a day (Bunting and his coworkers have not used orange juice); but the diets are essentially identical and we seem all to have been activated by a common desire to feed vitamins. Our own work has shown that, although caries appears usually to be arrested, other factors may enter in that must be taken into consideration. I refer to one case, one of three failures out of sixty-three cases to date, in which dental caries is unquestionably going on in spite of the diet. This woman is pregnant, she has had no end of worry and has lost considerable sleep. We know, of course, that worry and loss of sleep have a tremendous influence upon what we are pleased to call body

resistance. She may actually be in a state of vitamin deficiency even though she is ingesting plenty of food. We should not, perhaps, call this an exception but we cite this case as a brake to hyperenthusiasm and as a precaution to those who may be tempted to draw hasty conclusions. We cannot safely draw conclusions from any case until we have obtained every bit of available information, biochemical and psychological.

None of the work that has been done on the relation of diet to dental caries in the human subject is entirely free from criticism; but the indications are that caries can be arrested by proper feeding and with this very definite indication as a background it will probably not take long to ascertain the truth.

The Investigations of Mellanby, Pattison and Proud

In a series of three articles (23) that describe the results of experiments that have been conducted on about 125 tuberculous children, these authors come to some interesting conclusions. The summary to the last article reads as follows:

"In a batch of 21 children whose average age was about 5.5 years the addition of vitamin D in the form of irradiated ergosterol (radiostol) checked the initiation of new carious points, diminished the spread of old carious points and arrested the infective process in many carious teeth."

"This investigation corroborates those previously made, in which it was found that the carious process in the teeth of children could often be inhibited by increasing the intake of fat soluble vitamins by the addition to the diet of cod liver oil, egg yolk, and extra milk. The present work shows that vitamin D is a most powerful agent in these inhibitory processes."

All of these experiments have been conducted upon children ranging in age from 5 to 12 years. The children were afflicted with tuberculosis that had involved the bones and joints and, if we may judge from a few examples cited by Pattison (24), these children were quite sick and had been in poor health for some time. Pattison says,

"The children all suffered with tuberculosis of bones or joints, in which varying degrees of decalcification had occurred. It was found that in most cases those children who had suffered from the disease longest had the smallest amount of calcium in their saliva on admission to the hospital.

"The following are examples:

R.I. had had disease of the vertebrae for 10 years; it had produced marked bony destruction, and was active on admission; his saliva contained only 0.9 milligrams of calcium in 100 cubic centimeters.

H.L. had had tuberculosis of the sternum and tarsus for three years, with sinus formation; his saliva contained only 1.6 milligrams of calcium in 100 cubic centimeters.

N. A. had had active disease of the hip joint with abscess formation, but no bony destruction, for three months. His saliva contained 6.88 milligrams of calcium in 100 cubic centimeters."

Pattision says, moreover, "The average amount of calcium in the saliva of the children on

admission to hospital was only 4.77 milligrams in 100 cubic centimeters. This is low compared with the figures obtained by other workers, which vary between 6 and 12 milligrams per 100 cubic centimeters."

The experiments were, therefore, conducted on children that were in poor health and in which a disturbance in the calcium metabolism (probably also in the phosphorus metabolism) quite obviously existed. It is not surprising, under these conditions, that the addition of vitamin D to the diet increased the calcium content of the saliva (Pattison (24)) and this fact alone proves that vitamin D was certainly one of the constituents in which these children were markedly deficient. The addition of this substance to the diet would, of course, lead to a more perfect metabolic balance, would produce healthier body tissue and would tend to eliminate disease. A statement by Pattison is again very instructive in this connection:

"These patients were kept on diet B (less vitamin and more cereal) for four weeks only, as when this part of the investigation was attained the conclusion was reached that the general health of the patients on diet B was not so good as that of those on diet A"

Diet A (vitamins A and D rich) did, therefore improve the general health of the children probably because it supplied the very substances in which the tissues of these children was so markedly deficient.

It would be entirely fallacious to draw conclusions from these experiments that could be applied to normal children. Our own experiments have shown conclusively that at least the average American child between the ages of 11 and 17 is *not* in a state of vitamin D deficiency. I have seen cases of rampant caries in children who were ingesting two eggs and from one to two tablespoonfuls of cod liver oil per day and with a serum calcium of 12 and a serum phosphorus of 5. The important factor, as I see it, *is not some one constituent of the diet but a liberal ingestion of all of the essentials.*

Tuberculous children require considerable vitamin D and, when this essential constituent is added to the diet, an improvement occurs not only in general health but also in the condition of the teeth, The diet fed by Mellanby, Pattison and Proud was still deficient in vitamin C. *The carious process was retarded but it was not eliminated.* These children were still developing carious lesions. I question very much whether it would be possible, in such cases, to feed enough of anything to eliminate caries completely. Our own experience has indicated that infections of any kind are such a tax upon the human organism that food alone can scarcely maintain a normal metabolism. The results in these cases might, however, have been even better if the diet had been made more nearly complete by the addition of more vitamin C.

The Investigations of Boyd, Drain and Nelson (20)

These investigators have been recommending dietary essentials (for children) almost identical with those that we have recommended, namely, a quart of milk, one egg, a teaspoonful of cod liver oil, one ounce of butter, one orange, two or more servings of succulent vegetables and of fruits and such other foods as the child desires. The initial experiments were conducted on diabetic children; but these were soon extended to include normal healthy children. The dietary constituents, other than the above essentials, have been of the most diverse types. The calorie requirements of the one group were supplied largely by fat and of another group largely by carbohydrate. The results were essentially the same. Dental caries was arrested in all cases. A number of very important conclusions can be drawn from this investigation. The dietary essentials supply, as far as we now know, all of the minerals and vitamins that are required for the construction and maintence of healthy body tissue. The exact nature of the bulk of the food appears to be a matter of secondary importance in so far as the dental condition is concerned. Dental caries was arrested even when the major portion of the diet consisted of carbohydrate. This statement, which is in perfect accord with our own experience, calls for serious reflection on the part of the many dentists, dietitians and biochemists who have persistently maintained that dental caries is the direct result of the over ingestion of carbohydrate. Many people ingest carbohydrates to the exclusion of the essential foods and the deleterious results of such nutritional habits may be due to the lack of vitamins and minerals rather than to excessive ingestion of carbohydrate.

The diet used by these investigators is essentially identical with that which we have been advocating excepting that we have depended entirely (excepting in rare cases) upon eggs, milk and vegetables to supply vitamins A and D whereas the above investigators have used cod liver oil in conjunction with the above foods. We too have observed that caries can be arrested. The cod liver oil appears, therefore, (by a process of elimination) to be an unessential adjunct to the diet if the diet or the habits of the individual are such that they are already receiving an ample amount of vitamin D.

The Investigations of Bunting, Hadley, Jay and Hard (21)

These investigators believe that dental caries is the direct result of the action of organic acids upon the tooth structure. They have shown that an apparent relationship exists between dental caries and the prevalence

of *Bacillus acidophilus* in the mouth. They have also demonstrated that certain antiseptics such as metaphen and hexylresorcinol change the bacterial flora of the mouth so that *B. acidophilus* almost or quite disappears. They have attempted, in their most recent investigation, to correlate dental caries with diet and with the bacterial flora of the mouth.

This experiment, one of the most comprehensive that has been performed up to the present time, was conducted on children in a number of orphanages and on a group in a public school. The experiment included three groups that were fed "a well balanced, well fortified ration in which minimal amounts of sugar and white flour were included. Each child received one quart (946 cc) of milk and some green vegetables and fruit each day." Cod liver oil and viosterol were not administered. Hexylresorcinol was used as a mouth wash by two of these groups. The third group received no treatment other than the diet. Dental caries was practically arrested in all cases. The hexylresorcinol had no apparent virtue. *B. acidophilus* disappeared from the mouths of some but not from all of the children that had remained free from dental caries for one year.

Two additional groups of children served as controls. The diet was inadequate. The children in one of these groups used hexylresorcinol. Carious lesions developed in 50% of the children in *both groups*. The hexylresorcinol had no apparent virtue.

The significant observation is that a diet containing an ample supply of milk and fresh fruit and vegetables and restricted in carbohydrate does something that prevents the formation of carious lesions in the teeth of children. The exact nature of the fruits and vegetables and the quantities consumed is, unfortunately, not recorded by these investigators.

MILTON THEO. HANKE

REFERENCES

1. Krauterman, Sicherer Augen- und Kahnarst. 1732.
2. Bourdet, Recherches et observations sur toutes les parties de l'art duddentiste 1757. p. 95.
3. Findlay, G. M., The Blood and Blood Vessels in Guinea Pig Scurvy. *Jour. Path. and Bact.*, 1921, XXIV, 446.
4. Höjer, J. Axel. *Acta Paediat.*, III, 1923 *Supplementum*, 46; *Brit. Jour. Exper. Path.*, 1926, VII, 356.
5. Zilva, S. S. and Wells, F. M., Changes in the Teeth of the Guinea Pig Produced by a Scorbutic Diet. *Proc. Roy. Soc. of London, Series B*, XC, 505.
6. Howe, P. R., Effects of Scorbutic Diets upon the Teeth. *Dental Cosmos*, LXII, 586. Dental Caries. *Ibid.*, p. 921. The Effect of Vitamin Deficient Diet upon the Teeth. *Dental Cosmos*, 1921, p. 1086. Effect of Diet upon the Teeth and Bones, *Jour. Amer. Dent. Assoc.*, No. 3, 1923, p. 201. Food Accessory Factors in Relation to Teeth. *Jour. Dent. Research*, Mar. 1921.
7. Abbott, Frank., Caries of the Human Teeth. *Dental Cosmos*, 1879. Feb., Mar., April.

8. Heitzmann and Boedecker, Inflammation of Dentine (Eburnitis) *Indep. Pract.*, 1886. 120.
9. Miller, W. D., Micro-Organisms of the Human Mouth, 1890.
10. Excellent detailed anatomical descriptions can be found in "A Manual of Dental Anatomy" by Charles S. Tomes. 2nd Edition, 1882 and in "Dental Histology and Embryology" by B. Orban, Chicago, Ill., 1928.
11. "A Manual of Dental Anatomy." 2nd Edition. By Charles S. Tomes, 1882.
12. Bödecker, C. F. W., The distribution of living matter in human dentine, cement and enamel. *Dental Cosmos*, XX, 582–90, 645–657. A recent article by C. F. Bödecker, *Dental Cosmos*, LXXI, 586–98, contains a bibliography of the more recent work. This has been merely a confirmation of the older observations of C. F. W. Bödecker.
13. This matter is discussed in detail by C. S. Tomes (11).
14. This opinion has recently been voiced by Lyman A. Wilson. Is the calcification of dental enamel possible? *The Dental Digest*, XXXIV, 698–702. The work of other investigators is discussed in this paper.
15. Saliva ordinarily contains from five million to fifty million bacteria per cubic centimeter.
16. The teeth are painted with an alcoholic solution of bismarck brown (2% bismarck brown in 25% alcohol). The plaques appear as dark brown blotches or as a dark brown coating on the enamel. A clean enamel surface does not become stained.
17. I have been assisted in this work by a group of twelve Chicago dentists, members of the Chicago Dental Research Club.
18. Hanke, M. T., Relation of Diet to General Health and Particularly to Inflammation of the Oral Tissues and Dental Caries. *Jour. Amer. Dent. Assoc.*, XVII, 957–967.
19. Davis, Sherman., Nutritional Procedure for Arresting Caries. Report of Cases. *Jour. Amer. Dent. Assoc.*, XVII, 335–6.
20. Boyd, J. D. Drain, C. L. and Nelson, M. V., Dietary Control of Dental Caries. *Amer. Jour. Dis. Chil.*, XXXVIII, 721.
21. Bunting, R. W.; Hadley, Faith Palmerlee; Jay, Philip; and Hard, Dorothy G., The Problem of Dental Caries. *Amer. Jour. Dis. Chil.*, XL, No. 3, 536–548.
22. Rosebury, Theodor., A Biochemical Study of the Protein in Dental Enamel. *Jour. Dental Research:* 1930, X, 187.
23. Mellanby, M. and Pattison, C. L., *Brit. Med. Jour.*, 1924, 356; *Brit. Dent. Jour.*, 1926, XLVII, 1045; *Brit. Med. Jour.*, 1928, 1079.
24. Pattison, C. L., *Brit. Med. Jour.*, 1926, 6.
25. See also Black, G. V., The management of Enamel Margins, *Dental Cosmos.*, 1891, XXXIII, 1.
26. Bunting, R. W., and Palmerlee, F., *Jour. Amer. Dent. Assoc.*, 1925, XII, 381; Bunting, R. W., Nickerson, Gail and Hard, D. G., *Dental Cosmos*, 1926, LXVIII, 931.